Axotomy-Induced Upregulation of Tumor Necrosis Factor-Alpha in the Dorsal Root Ganglia

by

Pinhui Miao

A thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfillment of the requirements of the degree of

Master of Science

Faculty of Pharmacy
University of Manitoba
50 Sifton Road, Winnipeg, Manitoba
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Master's Thesis/Practicum Final Report

The undersigned certify that they have read the master's Thesis/Practicum entitled:

Axotomy-Induced Upregulation of Tumor Necrosis Factor-Alpha in the Dorsal Root Ganglia

Submitted by:

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In partial fulfillment of the requirements for the degree of:

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The Thesis/Practicum Examining Committee certifies that the thesis/practicum (and oral examination if required is:

Approved
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ABSTRACT

Neuropathic pain is a chronic pain syndrome associated with drug, injury or disease-induced destruction of sensory afferent fibers of the dorsal root ganglia (DRG). Although the exact underlying mechanisms involved in its pathogenesis is not known, pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α), is recognized as a principle modulator in the early development of neuropathic pain through inducing sensory neuronal apoptosis via the mitogen-activated protein kinases (MAPKs) pathway. The results of this study demonstrate a transient upregulation of TNF-α expression within bilateral DRG following unilateral sciatic nerve axotomy. Peak TNF-α expression is shown to occur within the first 7 days post-axotomy, which normalizes to baseline level by day 14. During the first week post-axotomy, the identified transient upregulation of TNF-α is associated with a switch in source production from satellite cells to sensory neurons. Our results indicate that peripheral nerve injury triggers the integral production of TNF-α within the DRG, representing a novel mechanism for axotomy-induced neuropathic pain.

KEY WORDS

DRG, neuropathic pain, sciatic nerve axotomy, cytokines, TNF-α, sensory neurons, satellite cells

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I still remember the first day of my Master's program. It was January 6, 2003, a windy day with beautiful sunshine, implying the start of a challenging, wondrous, and fruitful journey.

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LIST OF ABBREVIATIONS

5-HT: serotonin
AIDS: acquired immunodeficiency syndrome
AMPA: alpha-amino-3-hydroxy-5- methylisoxazole-4-propionic acid
APC: antigen presenting cell
ATP: adenosine 5'-triphosphate
BDNF: brain-derived neurotrophic factor
bFGF: basic fibroblast growth factor
bp: base pair
CGRP: calcitonin gene related peptide
cIAP: cellular inhibitor of apoptosis
dNTP: deoxynucleoside triphosphate
DRG: dorsal root ganglia
DTT: dithiothreitol
ERK: extracellular signal–regulated kinase
FADD: Fas-associated death domain protein
GABA: gamma-aminobutyric acid
GAPDH: glyceraldehyde-3-phosphate dehydrogenase
GDNF: glial cell line-derived neurotrophic factor
GFAP: glial fibrillary acidic protein
IB4: isolectin B4
IFN-γ: interferon-gamma
IFNGR1: interferon-gamma receptor I
IFNGR2: interferon-gamma receptor II
IKK: inhibitor of κB kinase
IL-12: interleukin-12
IU: international unit
JNK: c-Jun N-terminal kinase
MAPK: mitogen-activated protein kinase
mGluR: metabotropic glutamate receptor
MHC II: major histocompatibility complex II
M-MLV RT: moloney-murine leukemia virus reverse transcriptase
MS: multiple sclerosis
NE: norepinephrine
NF-κB: nuclear factor-κB
NGF: nerve growth factor
NK cell: natural killer cell
NMDA: N-methyl-D-aspartate
NPY: neuropeptide Y
NT-3: neurotrophin-3
PBS: phosphate buffer saline
PBS-T: phosphate buffer saline-Triton

PCR: polymerase chain reaction
PNI: peripheral nerve lesion
PNS: peripheral nervous system
Real-time PCR: real-time polymerase chain reaction
RIP1: receptor-interacting protein type 1
RRMS: relapsing-remitting multiple sclerosis
RT: reverse transcriptase
RT-PCR: reverse transcriptase-polymerase chain reaction
SD: standard deviation
SMT: spinomesencephalic tract
SP: substance P
SRT: spinoreticular tract
STT: spinothalamic tract
TBE: Tris-borate-EDTA
TCA: tricyclic antidepressant
TGF: transforming growth factor
TGF-β1: transforming growth factor-β1
Th1 cell: type I T helper cell
TMP: thiamine monophosphatase
TNF-α: tumor necrosis factor-alpha
TNFR1: tumor necrosis factor receptor 1
TNFR2: tumor necrosis factor receptor 2
TRADD: tumor necrosis factor receptor-associated death domain
TRAF2: tumor necrosis factor receptor-associated factor 2
trkA: tyrosine kinase A
trkB: tyrosine kinase B
trkC: tyrosine kinase C
TRPV2: transient receptor potential vanilloid 2
UV: ultraviolet
VR1: vanilloid receptor 1

1. BACKGROUND

Neuropathic pain occurs in approximately 1% of the population (Stacey 2005). It constitutes approximately 25-50% of all pain clinic visits (Verma et al. 2005). Characteristic clinical presentations of neuropathic pain includes sensory abnormalities such as the feeling of pins and needles, burning, shooting and/or stabbing pain with or without throbbing and numbness (Jensen et al. 2001; Namaka et al. 2004). Although the etiology of neuropathic pain has been extensively studied, the exact underlying mechanism involved in its pathogenesis is still not known. Irrespective, it is recognized as a chronic pain syndrome associated with drug, injury or disease-induced destruction of sensory afferent fibers of the dorsal root ganglia (DRG).

Various drugs have been associated with the development of neuropathic pain. For example, drugs such as isoniazid, ethambutol, ethionamide, nitrofurantoin, itraconazole, metronidazol, vinca alkaloids, perhexiline, hydrallazine, methaqualon, indomethacin, chloroquine, phenytoin, as well as interferon-alpha, statins, cisplatin, suramin, and tacrolimus have all been reported to be associated as potential causative agents involved in neuropathic pain (Argov and Mastaglia 1979; Peltier and Russell 2002; Vardizer et al. 2003; Chong et al. 2004; Pratt and Weimer 2005; Singh and Cundy 2005). In addition, heavy metals and alcohol have also been reported to cause neuropathic pain (Koike et al. 2001; Rubens et al. 2001; Eaton and Qian 2002; Hoffman 2003).

Physical injury is another common cause of neuropathic pain. For example, automobile accidents, falls, surgeries, and sports-related injuries associated partial or complete nerve

damage (severed, crushed, compressed, or stretched) have all been linked to the development of chronic neuropathic pain syndromes (Elman and McCluskey 2004; Sarkies 2004; Harden 2005; Nienhuijs et al. 2005).

Furthermore, a variety of diseases, such as acquired immunodeficiency syndrome (AIDS), diabetes, herpes zoster, Guillain-Barre syndrome, and multiple sclerosis (MS), have also been associated with induction of neuropathic pain (Moulin 1998; Lilie and Wassilew 2003; Lopez et al. 2004; Aaron et al. 2005; Kelkar 2005; Lewis 2005).

Irrespective of its cause, disruption of sensory homeostatic mechanisms in the DRG of the peripheral nervous system (PNS) is recognized as a common target involved in chronic neuropathic pain.

1.1 *DRG*

DRG are sensory ganglia that lie within the vertebral column immediately adjacent to the spinal cord (Kandel 1991). The DRG house a variety of distinct non-neuronal and neuronal cell types (Devor 1999; Verge et al. 2004; Zerboni et al. 2005; Kelm et al. 2006).

1.1.1 *Microglia/Macroglia*

In general, there are two major classes of non-neuronal cells integral to the structure and function of DRG which include microglia and macroglia (Ono et al. 1999; Abbadie et al. 2003; Lam et al. 2003).

Specifically, the microglia are phagocytes that become mobilized in case of injury, infection or disease (Carson et al. 2004). Phagocytes defend the host through non-specific immune response mechanisms. The main functions of phagocytes are to ingest and digest invading microorganisms (Beers 1999). Phagocytes include a variety of cell types such as neutrophils, monocytes (in the blood) and macrophages (in the tissues) (Kasama et al. 2005). Widely distributed, macrophages are strategically situated at the interfaces between tissues and blood where they recognize and bind to foreign substances to the body. The protective mechanism by which macrophages exert their effect is attributed to their ability to express major histocompatibility complex II (MHC II). MHC II expression on the surface of macrophages plays a critical role in recognizing foreign antigens that enter the body (Hu and McLachlan 2003). As a result, they are considered as antigen presenting cells (APCs) that are essential to immune system activation (Bomstein et al. 2003). Macrophages have been identified within the DRG by the presence of EBM-11 and Leu-M3 on their cells surface (Graus et al. 1990). Macrophages have been subdivided into several main morphological subtypes that include: ramified, ameboid, and bipolar phenotypes (Hutchins et al. 1992; Williams et al. 1992; Roggendorf et al. 1996). Positionally within the DRG, macrophages are often located between the macroglial cells such as satellite cells that can also function as APCs (Fenzi et al. 2001).

1.1.2 *Satellite cells*

Satellite cells are small macroglial cells that display a flat appearance localized in close proximity to the circumference of sensory neurons. The mean diameter of satellite cells

located in rat DRG is approximately 7.2-7.5μm (Cecchini et al. 1999). They provide nutritive support to the neurons and other cell types housed within the DRG in paracrine fashion, by secreting neurotrophic factors such as nerve growth factor (NGF) and neurotrophin-3 (NT-3) essential for their survival (Jongsma Wallin et al. 2001; Dodge et al. 2002; Liu et al. 2002). In addition to their morphological characteristics and positional distribution around sensory neurons, the satellite cells can also be identified by the presence of distinct immunohistochemical markers, such as glial fibrillary acidic protein (GFAP) (Peters et al. 2005), and S-100 (Gonzalez-Martinez et al. 2003). Besides their sustaining and nutritive function within DRG, satellite cells also express MHC II, thereby providing a protective function as APC. Specifically, satellite cells are known to protect sensory neurons from pathogenic invasion of various bacteria and viruses (Graus et al. 1990). In addition to satellite cells, other macroglial cells also located within the DRG include fibroblasts and Schwann cells.

1.1.3 *Fibroblasts*

Fibroblasts represent another type of macroglial cell type found within the DRG. Analogous to satellite cells, these connective cells lie interneuronally within DRG. Morphologically, they are flat in appearance and present many hillocks on their cell bodies (Kandel 1991), with diameter of their cell bodies approximately 10-20μm (van Dorp et al. 1990). Fibroblasts are involved in collagen production used to form the fibrillar matrix of connective tissue, thereby providing firmness and structural support to the DRG (Yang et al. 1999). Similar to satellite cells, fibroblasts also sustain, support, and protect sensory neurons by producing various

nutritive substances, such as: brain-derived neurotrophic factor (BDNF), NGF, and basic fibroblast growth factor (bFGF) (Frim et al. 1993; Zhou et al. 1999; Jin et al. 2002; Liu et al. 2003), that are essential for the survival and maintenance of the neurons housed within DRG. Besides their morphological characteristics, fibroblasts can be identified using immunohistochemistry that recognize specific markers expressed on their cell surface, such as vimentin and fibronectin (Dubovy et al. 2002; Conrad et al. 2005).

1.1.4 *Schwann cells*

Schwann cells represent the third major class of macroglial cells found within the DRG. These cells wrap along the length of a single neuronal axon in a three-dimension-spiral fashion, the resultant myelin sheath is essential for electrical nerve impulse transmission and propagation that governs normal bodily functions (Oguievetskaia 2005). Each Schwann cell makes up a single segment of a neuronal axon's myelin sheath (Kingsley 1999). The intervals between the segments of myelin, which is about 1mm long, are termed the nodes of Ranvier (Poliak and Peles 2003; Sherman and Brophy 2005). The thickness and presence or absence of the myelin coating largely depends on the subtype of sensory neuron from which axons evolve (Tjen et al. 2005). Similar to satellite cells and fibroblasts, Schwann cells are also important supporting cells of sensory neurons housed within DRG. They secrete neurotrophins, such as: NGF, NT-3, NT-4 (Frostick et al. 1998; Hansen et al. 2001; Hiroi et al. 2005), and other substances, such as: insulin-like growth factors, platelet derived growth factor (Eccleston et al. 1993; Fushimi and Shirabe 2004), that are integral to the survival of sensory neurons. Schwann cells can be identified through the use of several well known

immunohistochemical markers that include: S-100, fast blue, and cellular prion protein. (Bergers et al. 2002; Dedkov et al. 2002; Ford et al. 2002).

1.1.5 *Sensory neurons*

Morphologically, the sensory neurons within the DRG are composed of cell body, dendrites, and axon (Kandel 1991). Sensory neurons are pseudo-unipolar neurons that have a peripheral and a central projection. The peripheral projection connects to skin, muscle, or joints, while the central projection enters the spinal cord at the dorsal tip of the dorsal horn (Martin 2003). The peripheral and central projections of DRG neurons are called primary afferent fibers. These afferent fibers convey a variety of different sensory modalities from the external environment, such as: touch, pain, temperature, proprioception, and vibration, to the DRG and eventually relay these information centrally for higher order processing (McGraw et al. 2005).

DRG neurons have been classified according to various methods that include: neuronal diameter, axonal diameter, presence or absence of myelin, as well as electrical impulses conduction velocity (Windebank et al. 1985; Djouhri et al. 2003).

There are three main subpopulations of primary afferent fibers of DRG neurons: Aβ, Aδ, and C. Aβ fibers are thick myelinated nerve fibers of large-sized neurons (soma diameter>45μm) that convey non-noxious, low intensity mechanical stimulus sensory information at an accelerated rate of 35-120m/s. Aδ fibers are thinly myelinated nerve fibers of smaller-sized

neurons (soma diameter=30-45μm) that have a intermediate conduction velocity of 5-30m/s. C fibers are unmyelinated nerve fibers of smallest-sized neurons (soma diameter<30μm) which have the slowest conduction velocity of 0.5-2m/s (Kandel 1991; Lawson 2002; Fang et al. 2005; Ma and LaMotte 2005).

All three types of afferent fibers are able to convey non-nociceptive information, but only Aδ and C fibers are able to convey nociceptive information. Aβ fibers predominantly transmit fast impulses, including touch, movement, vibration, and pressure, Aδ and C fibers are responsible for slower impulses and pain transmission (Fang et al. 2005). The sensory neurons that give rise to Aδ and C fibers comprise approximately 70% of all neurons within the DRG (Snider and McMahon 1998). Both Aδ and C fibers transmit noxious mechanical, chemical, and thermal stimuli, the main difference between them is that C fibers are unmyelinated, Aδ fibers are myelinated, as a result, pain transmitted by Aδ fibers is clinically described as very sharp and well localized, while C fibers pain is dull and poorly localized (Narhi et al. 1992; Ngassapa 1996).

DRG neurons have also been subdivided according to their own inherent specificity for various neurotrophic factors. Neurotrophins are a family of peptides that regulate the growth, proliferation, differentiation, and survival of DRG neurons. NGF, BDNF, NT-3, and NT-4 are several examples of such neurotrophins, however, glial cell line-derived neurotrophic factor (GDNF) represents a non-neurotrophic factor utilized by select neuronal subpopulations (Paves and Saarma 1997; Wang et al. 2003).

At present, there are six main subtypes of neurotrophin specific DRG neurons that have been identified: tyrosine kinase A (trkA)/NGF, tyrosine kinase B (trkB)/BDNF, trkB/NT-4/5, tyrosine kinase C (trkC)/NT-3, Ret/GDNF, and P2X3/ATP dependent neurons (Vulchanova et al. 1998; Karchewski et al. 1999; Josephson et al. 2001).

1.1.5.1 *TrkA/NGF dependent neurons*

Some neurons within DRG are predominately responsive to NGF. This process is usually mediated by a high affinity trkA receptor and a low affinity P75 receptor (Carroll et al. 1992; Murray and Cheema 2003). The interaction of NGF with the complex of trkA/P75 results in the high affinity binding of NGF essential for signaling and other biological activity (Canossa et al. 1996). The NGF responsive neurons are usually small to medium soma diameter neurons, with size less than 30μm (Shu and Mendell 2001). As a result, the sensory afferent fibers that project from these sensory neurons are often unmyelinated or thinly myelinated (Silos-Santiago et al. 1995), which correspond to C and Aδ fibers, respectively. Hence, TrkA/NGF dependent DRG neurons convey nociceptive sensory information, such as: thermal and mechanical information, and display characteristic immunohistochemical markers such as: calcitonin gene related peptide (CGRP), and substance P (SP), which are known nociceptive neurotransmitters (Lawson et al. 1996; Pezet et al. 1999). For instance, following peripheral nerve ligation, the number of trkA/NGF responsive neurons in the DRG slowly declined during the first three weeks, which was followed by a full recovery at two months (Shen et al. 1999). The early decrease of trkA/NGF responsive neurons number is likely due to deprivation of target-derived NGF caused by nerve ligation. The subsequent

recovery might either be because alternative sources of NGF become available or neurogenesis occurs (Namaka et al. 2001). The predominant effects of nerve ligation on trkA/NGF responsive neurons are not surprising as these are the neurons responsible for the transmission of nociceptive information induced by ligation. This evidence indicates that although trkA/NGF neurons in the DRG initially adapt themselves to survive in response to injury, the initial protection may trigger downstream events involved in the development of injury-induced chronic pain syndromes due to the preferential loss of Aδ and C fibers.

1.1.5.2 *TrkB/BDNF and trkB/NT-4/5 dependent neurons*

Both BDNF and NT4/5 responsive neurons bind to high affinity trkB receptors, as well as low affinity P75 receptors to exert their maximal biological effects (Kalb 2005; Williams et al. 2005). TrkB positive neurons in DRG are predominately medium-sized, ranging in size from 25μm to 40μm in soma diameter (Mu et al. 1993). They usually possess thinly myelinated afferent fibers, which are also termed Aδ fibers (Mu et al. 1993). These afferent fibers convey nociceptive information, such as sharp and well-localized pain from the periphery to the CNS (Kandel 1991). Approximately 50% of the TrkB dependent neurons show immunoreactivity for neuropeptide Y (NPY) (Gorba and Wahle 1999). Interestingly, following a focal crush injury of the sciatic nerve, trkB responsive neurons survive throughout the post-injury time course (Sebert and Shooter 1993), suggesting that trkB responsive neurons are less prone to the damaging effects induced by peripheral nerve injury. In fact, injury induces these neurons to synthesize BDNF within the DRG which may assist in the survival and maintenance of themselves.

1.1.5.3 *TrkC/NT-3 dependent neurons*

Another main subpopulation of DRG neurons includes that of the NT-3 responsive subtype, which predominantly requires NT-3 for survival, development, and maintenance (Lefcort et al. 1996). This particular subpopulation is composed of the neuronal cells that range in soma diameter from medium (25-40μm) to large (>40μm) (Mu et al. 1993). Axonal projections from these sensory neurons are often thickly myelinated Aβ and Aδ afferent fibers (Kandel 1991). NT-3 is considered to bind to the high affinity trkC receptor and the low affinity P75 receptor to exert its maximal biological effects (Friedman and Greene 1999; Zaccaro et al. 2001). However, Wright and Snider reported in 1995 that P75 is coexpressed in only 50% of trkC responsive neurons, suggesting a potential new sensory neuronal subtype characterized by trkC dependence and P75 independence (Wright and Snider 1995). Additional research into this area is required before any definite conclusion can be drawn. TrkC/NT-3 dependent neurons show immunoreactivity for the ionotropic purine receptors P2X4, P2X5, and P2X6 (Kobayashi et al. 2005), and transient receptor potential vanilloid ion channels subfamily member 2 (TRPV2) (Tamura et al. 2005). TrkC/NT-3 dependent neurons usually convey non-nociceptive, rapidly transmitted sensory information, such as: touch, proprioception, pressure, and vibration (Kandel 1991). Confirmation of their functions has been obtained from study on trkC-deficient mice that have displayed abnormal movements consistent with loss of proprioception (Barbacid 1994). Another *in vivo* study demonstrated that after unilateral dorsal root rhizotomy in cat, the number of trkC/NT-3 dependent neurons (>57μm) in the DRG decreases, while the number of small size neurons (<42μm) significantly increases (Li et al. 2000). However, the same group of scientists reported conflicting results

stating that the number of large trkC/NT-3 dependent neurons (57-100μm) increases following peripheral injury (Ni et al. 2001). Against this confusion, other researchers have shown that NT-3 expression is not even detected either in intact or nerve injured rat DRG (Shen et al. 1999), indicating a lack of injury-induced response on this specific sensory neuronal phenotype. Although there are conflicting findings of these studies, in general, large diameter trkC/NT-3 dependent neurons are predominantly unaffected by injury (Frisen et al. 1993; Liebl et al. 2001; Cui et al. 2002).

1.1.5.4 *Ret/GDNF dependent neurons*

The DRG also contains another neuronal subtype identified by the presence of GDNF receptor and trk receptor: Ret (Zihlmann et al. 2005). The Ret/GDNF dependent sensory neurons predominately have cell bodies in small to medium diameter (<25μm), and thereby transmit nociceptive sensory information (Honda et al. 1999). Their corresponding axons are usually unmyelinated or thinly myelinated, but are uniquely different from the previously described trkA responsive neurons (Leitner et al. 1999). GDNF is not a member of the neurotrophin family, but a member of the transforming growth factor (TGF) super family (Chiang et al. 2005). However, analogous to neurotrophins, GDNF must bind to a high affinity receptor GDNFR to form a complex and then interacts with Ret to exert its maximal biological effects (Treanor et al. 1996; Klein et al. 1997; Sanicola et al. 1997). The Ret/GDNF dependent neurons are known to be immunoreactive for isolectin B4 (IB4), as well as an enzyme called thiamine monophosphatase (TMP) (Bennett et al. 1998).

In 1998, Bar *et al* reported that higher levels of GDNF were found in DRG axotomized from the spinal cord than in post-mortem control DRG. As a result, this neuronal subtype doesn't appear to be very affected by injury to the same degree as the TrkA/NGF responsive neurons. In addition, GDNF immunostaining was seen in Schwann cells and in DRG neurons, especially of small and medium size cells, while that of Ret was restricted to DRG neurons and axons, with no significant changes in numbers of positive DRG neurons after injury (Bar et al. 1998). These findings suggest that the Ret/GDNF dependent neurons may be self-supported in autocrine fashion in case of peripheral injury instead of relying on central or peripheral target for neurotrophic support.

1.1.5.5 *P2X3/ATP dependent neurons*

This subpopulation of DRG neurons contains thr adenosine 5'-triphosphate (ATP) receptor, P2X3. The P2X3/ATP dependent neurons in the DRG are predominately small sized (<25μm), and their axons correspond to C fibers, which are involved in nociceptive information transmission (Novakovic et al. 1999; Kage et al. 2002). Approximately 40% of neuronal profiles in normal rats DRG are P2X3/ATP responsive (Vulchanova et al. 1998), most of them are also GDNF sensitive (Ramer et al. 2001). P2X3/ATP responsive neurons show immunoreactivity to neurofilament (Petruska et al. 2000). P2X3/ATP responsive neurons usually convey pain information from periphery to CNS, therefore, to selectively block the P2X3 receptor might be significant in achieving pain relief (Honore et al. 2002). Immunohistochemical studies indicate that the number of P2X3/ATP positive neurons in rat DRG is increased following neuropathic injury induced by chronic constriction of the sciatic

nerve (Novakovic et al. 1999). Other studies, however, indicate that levels of P2X3 receptor expression were decreased in ipsilateral DRG after peripheral axotomy in the rat (Bradbury et al. 1998; Kage et al. 2002). Interestingly, it was also shown that GDNF may completely reverse axotomy-induced downregulation of the P2X3 receptor (Bradbury et al. 1998), implying that the expression of P2X3 receptor in DRG after peripheral injury can be regulated by other factors.

It has already been identified that the approximate percentages of human adult DRG neurons expressing p75, trkA, trkB, trkC or Ret is 57%, 46%, 29%, 24% and 79%, respectively (Josephson et al. 2001). Another quantitative *in situ* hybridization study in human adult shows a slightly different distribution of neuronal subtypes: 79%, 41%, 33%, 43% for p75, trkA, trkB, trkC, respectively (Karchewski et al. 1999). Co-localization studies reveal that approximately 10% of DRG neurons co-express trkA and trkB mRNA, 19% co-express trkA and trkC mRNA, and 18% co-express trkB and trkC mRNA, while tri-localization of all three trk mRNAs is approximately 3-4% of neurons in the DRG (Karchewski et al. 1999). The obvious overlap among the neuronal subtypes may serve to inherently protect the sensory neurons by allowing them to change neurotrophic dependence in response to injury in order to minimize neuronal loss. This unique ability identifies the extreme plasticity of DRG neuronal cells.

Besides these major classifications of DRG sensory neurons, there are other newfound categories that include non-peptide-expressing neurons and P-neurons (Acosta et al. 2001;

Priestley et al. 2002). Non-peptide-expressing neurons are small sized, immunoreactive for vanilloid receptor 1 (VR1), and usually convey thermal nociceptive information (Priestley et al. 2002). P-neurons are small diameter in soma, projecting unmyelinated fibers (Acosta et al. 2001). They are immunoactive to neurofilament of 200kDa, and neither NT-3 nor BDNF influence their survival during embryonic and postnatal stages, respectively (Acosta et al. 2001). Most interestingly, embryonic P-neurons require NGF, but not bFGF, while postnatal P-neurons need bFGF but not NGF for survival *in vitro* (Acosta et al. 2001). This trophic switch during different early developmental stages is unique to that of DRG neuronal subpopulations.

1.2 *Pain transmission*

1.2.1 *Pain processing loop*

Painful stimuli from periphery are transmitted via sensory afferent fibers (Aδ and C fibers) to a collection of sensory neuron cell bodies within DRG. The nociceptive afferent impulses received by the DRG are then transmitted centrally via dorsal roots to the main pain processing areas of the spinal cord in the superficial laminae I and II. The afferent impulse is then relayed centrally via the ascending pathways, including spinothalamic tract (STT), spinoreticular tract (SRT), and spinomesencephalic tract (SMT), for higher order processing. The pain processing center (mainly cortex) then activates descending pain-control pathways to release various neurotransmitters such as norepinephrine (NE), serotonin (5-HT), endorphin, NPY, and gamma-aminobutyric acid (GABA), which trigger a complex cascade of interactions that ultimately inhibit the excitatory transmission that originated at the level of

spinal cord (Przewlocki and Przewlocka 2001; Moran et al. 2004). Overall, the net result is the formation of an entire pain-processing loop driven by nociceptive afferent fiber input that is eventually suppressed by a descending antinociceptive output (Millan 1999; Namaka et al. 2004)

1.2.2 *Ascending pathways*

Incoming peripheral afferent fibers enter the spinal cord via the DRG, where they synapse in the dorsal horn. The sensory information is then conveyed via various ascending pathways for higher order processing in the brain. Specifically, the non-nociceptive sensory information is transmitted through the dorsal column-medial lemniscal system, while noxious information is mediated through the anterolateral system (Kandel 1991; Saade et al. 2002). There are three major pathways of the anterolateral system: STT, SRT, and SMT (Lynn 1992; Mense 2004).

STT is the most important and mostly studied pathway. It is the major ascending pathway relaying specific information as to the intensity, location, duration, rate, and quality of the noxious stimuli (Willis 1985; Zhang and Giesler 2005). Both Aδ and C fibers mainly project in the superficial dorsal horn at lamina I (marginal zone) and lamina II (substantia gelatinosa) (Dubuisson 1989). Pain information from lamina I and II is then transmitted to interneurons in deeper laminae of the spinal cord (Tavares and Lima 2002). The axons of these interneurons in deeper laminae cross the midline and ascend all the way to the thalamus and cortex in the anterolateral quadrant of the contralateral half of the spinal cord for higher

order processing (Ralston and Ralston 1992).

Besides STT, there are SRT and SMT, which are also involved in pain transmission (Mense 2004). SRT originate primarily in laminaⅦ and Ⅷ of the spinal cord (Willis 1985). They terminate in the brain stem reticular formation, and further project to many areas of the brain, including the hypothalamus and the thalamus (Willis and Westlund 1997). SRT relays nociceptive information contributing to the motivational and affective responses aspects of pain (Chapman et al. 1985). SMT neurons originate in lamina Ⅰ, Ⅳ, and Ⅴ in the dorsal horn of the spinal cord (Wiberg and Blomqvist 1984; Fleetwood-Walker et al. 1988). They terminate in the midbrain (Lynn 1992). SMT plays a critical role in relaying and integrating nociceptive information that produce affective and aversive behaviors such as fear (Yezierski and Broton 1991). Activation of these ascending pain pathways subsequently activate the descending inhibitory pathways which produce endogenous analgesia.

1.2.3 *Descending pathways*

There are five major descending pathways have been identified, corticospinal, rubrospinal, reticulospinal, vestibulospinal, and tectospinal tract (ten Donkelaar 2000; Chen et al. 2002; Pettersson and Perfiliev 2002).

The corticospinal tract is the largest and most important descending pathway (Kandel 1991; Canedo 1997). The neurons of the corticospinal tract originate from the cerebral cortex (Coonan et al. 2001; Cincotta et al. 2003). As the tract descends, the corticospinal tract is

divided into two bundles, approximate 90% of the fibers traverse the medulla within a well-defined pyramidal tract, decussates within several fascicles at the spinomedullary junction, and extends down the spinal cord in a compact bundle in the dorsal funiculus (Stanfield 1991; Joosten et al. 1992). The remaining 10% of the descending fibers continue to descend ipsilaterally without decussation (Armand and Kuypers 1980). Most corticospinal tract axons terminated in lamina□ and □ of cervical segments of the spinal cord (Yang and Lemon 2003).

The rubrospinal tract neurons have their cell bodies in the red nucleus, which is an encapsulated cell group situated in the tegmentum of the midbrain (Murray and Gurule 1979; Kwon et al. 2004). Their fibres decussate immediately on leaving the red nucleus and descend through the pons, medulla and spinal cord as the tract descends (Wild et al. 1979; Raineteau et al. 2002), and further terminate on interneurons in lamina V and VI through the entire length of the spinal cord (Kuchler et al. 2002).

The reticulospinal tract neurons can be divided to two subgroups, one has the cell bodies in the pontine reticular formation, while another group of neurons has their cell bodies in the medullary reticular formation (Sholomenko and O'Donovan 1995). The fibres from the pons descend in the pontine reticulospinal tract, and are mainly ipsilateral, terminating in laminaⅦ and Ⅷ of the spinal cord (Matsuyama et al. 1999). The fibres from the medulla descend in the medullary reticulospinal tract, and are both ipsilateral and contralateral, terminating among the gray matter in lamina Ⅴ-Ⅷ (Perreault et al. 1993; Takakusaki et al. 1994).

The vestibulospinal tract neurons have their cell bodies in the vestibular nuclei situated in the pons (Nathan et al. 1996; Boyle and Johanson 2003). There are two subgroups of the neurons. Some neurons have their cell bodies in lateral vestibular nuclei, whose axons descend the length of the spinal cord ipsilaterally without decussation, and enter laminae Ⅶ and Ⅷ of the anterior horn (Kuze et al. 1999; Tellegen et al. 2001). Other neurons have their cell bodies in the medial vestibular nuclei, with axons descending ipsilaterally only to cervical levels, terminating mainly in lamina Ⅶ-Ⅸ (Shinoda et al. 1989; 1992; Shinoda et al. 2005)

The tectospinal tract neurons have their cell bodies in the tectum. Their fibers mostly decussate just caudally to their origin in the midbrain, and descend through the midbrain, pons and medulla, terminating on medially placed interneurons within the cervical spinal cord (Dicke 1999).

The descending pathways inhibit ascending pain transmission at the level of spinal cord via ionic and neurotransmitter mechanisms (Holmes and Fujimoto 1994; Bruce et al. 2002; Hasue et al. 2004; Namaka et al. 2004). In either case, ion channels are altered as to their number and location for the sole purpose of downregulating the states of neuronal hyperexcitability (Pasero 2004; Dobremez et al. 2005). Both ionic and neurotransmitter mechanisms play a crucial role in the underlying pathophysiology of neuropathic pain.

1.3 *Pathophysiology*

The pathophysiological mechanisms involved in neuropathic pain are not completely

understood but are considered to be complex, multifactoral, and to evolve over time (Chevlen et al. 2005). Current studies indicate that the cellular changes results from ionic and neurotransmitter mechanisms contribute to the pathogenesis of neuropathic pain (Neale et al. 2005).

1.3.1 *Ionic mechanism*

Neuronal hyperexcitability is the hallmark cellular characteristic of neuropathic pain (Jensen 2002). Neuronal hyperexcitability is produced by increased excitation and/or decreased inhibition, which result from changes of ion permeability (Jen et al. 2005). Ions such as sodium (Na^+), calcium (Ca^{2+}), potassium (K^+), and chloride (Cl^-) are involved in altering the cellular membrane potential (Busse et al. 2005; Shin et al. 2005). During an event of drug-, disease-, or injury-induced damage or destruction of peripheral nerve, neuronal cells become excited and Na^+ channels start to open, resulting in an influx of Na^+ into the cell, and subsequently trigger an influx of Ca^{2+} via reversal of the Na^+-Ca^{2+} exchanger, which further alters the existing electrolyte concentration gradients (Fung 2000; Okuyama et al. 2000; Brown et al. 2001; Wolf et al. 2001). This depolarizing response will result in K^+ channels being opened, the K^+ efflux begins to counteract the influx of positive ions (Na^+ and Ca^{2+}), bringing the membrane potential back toward the original resting state (Bhattacharjee and Kaczmarek 2005). Hence, any defects in the homeostatic regulation of these ions may lead to enhanced states of neuronal hyperexcitability.

1.3.2 *Neurotransmitter mechanism*

In conjunction with the ionic mechanism, neurotransmitters play a key role in the development of neuronal hyperexcitability. During the resting state, there is a balance existing between excitatory and inhibitory neurotransmitters (Dickenson et al. 1997). Glutamate, a widely studied excitatory neurotransmitter, exerts its excitatory effects by its ability to bind to two major ionotropic receptors, alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) and N-methyl-D-aspartate (NMDA), on the cellular membrane (Barnes and Slevin 2003). During a triggering event, released glutamate is able to bind to both AMPA and NMDA receptors, which are involved in the opening of Na^+ and Ca^{2+} channels. The influx of these cations results in an excitatory depolarizing response of the neuronal cell (Martinez-Sanchez et al. 2004). Besides ionotropic receptors, there are three groups of G-protein-coupled metabotropic glutamate receptors (mGluR) that modulate neuronal excitability (Meldrum 2000). mGluRs can be further subdivided into eight subtypes (mGluR1-8) based on sequence homologies, mechanisms of signal transduction as well as pharmacological characteristics (Ritzen et al. 2005). Group I mGluRs consists of mGluR1 and 5, group II consists of mGluR2 and 3, while groupIII includes mGluR4, 6, 7, 8 (Dolan et al. 2004; Guo and Ikeda 2005). In general, group I mGluRs mediate excitatory effects, whereas group II and III decrease synaptic transmission and usually induce inhibitory effects on neuronal excitation (Lanneau et al. 2002; Yang and Gereau 2003; Chen et al. 2005; Chen and Pan 2005). Regardless the different roles that mGluR subtypes play in the development of neuronal hyperexcitability, glutamate is generally recognized an excitatory neurotransmitter.

In contrast, inhibitory neurotransmitters such as GABA attempt to restore the normal balance between excitation and inhibition (Ferraro and Sardo 2004). GABA participates in the inhibition of neuronal hyperexcitability through interaction with $GABA_A$ and $GABA_B$ receptors on the cellular surface (Johnston et al. 2001). The binding of GABA to $GABA_A$ receptors produces fast synaptic inhibition through the opening of Cl^- channels, the influx of anions into the cell suppresses cellular excitation (Hevers and Luddens 1998). GABA can also bind to $GABA_B$ receptors to exert its inhibitory effect. The interaction of GABA with $GABA_B$ receptors works through a G-protein-coupled receptor to facilitate the opening of K^+ channels and the closure of Ca^{2+} channels (Fearon et al. 2003; Richman et al. 2004). Both events result in the production of a more negative membrane potential, resulting in an inhibitory response.

Additionally, endorphins, enkephalins, dynorphins, NE, 5-HT, and NPY are also known to be inhibitory neurotransmitters (Delgado 2004; Holden et al. 2005; Li et al. 2005). Therefore, activation of descending pathways that release these types of anti-nociceptive neurotransmitters suppresses incoming neuronal hyperexcitability at the dorsal horn level of the spinal cord.

Many drugs that effectively treat neuropathic pain, such as opioids and antidepressants, mimic the action of the inhibitory transmitter system associated with the descending antinociceptive pathways, to produce analgesia (Brown and Bottomley 1990; Advokat 1993). For example, tricyclic antidepressants (TCAs) are believed to alleviate neuropathic pain

predominately through blocking the reuptake of anti-nociceptive neurotransmitters, such as NE and 5-HT, in the CNS descending pathways from which they are released (Barkin and Fawcett 2000). As a result, TCAs have been extensively used in the treatment of a variety of neuropathic pain disorders, and are considered first-line agents in the pharmacologic management of neuropathic pain (Hemstreet and Lapointe 2001; Namaka et al. 2004; Saarto and Wiffen 2005).

Although effective pharmacologic therapy is available to assist in the management of neuropathic pain, current therapy is still inadequate at alleviating pain (Dray 2004). At best, current treatment strategies only reduce pain to a tolerable level (Namaka et al. 2004). The lack of effective therapeutic agents for neuropathic pain is due to the fact that its underlying pathophysiological mechanisms are still not fully understood.

However, recent studies indicate that the transmission of the nociceptive and antinociceptive information depends upon the activation of cell signaling pathways of inflammatory responses, which are orchestrated by cytokines (White et al. 2005).

1.4 *Cytokines*

Cytokines are soluble proteins or glycoproteins that were originally described to mediate activation of the immune system and inflammatory responses (Sommer 2001). Cytokines are secreted by T-cells and a variety of other cells including those in the nervous system (Moalem et al. 2004). They are usually produced in response to local stimuli, such as injury,

the presence of antigens or endotoxins, or the transduction of signals provided by other cytokines (Cunha et al. 2000). Cytokines can be divided into two subgroups: pro-inflammatory and anti-inflammatory cytokines. In order to maintain homeostasis, a dynamic balance must be struck between pro- and anti-inflammatory cytokines (Milligan et al. 2005). Upregulation of pro-inflammatory cytokines may result in excess inflammation, which may further cause a variety of diseases, as in the case of neuropathic pain (Ma and Quirion 2005). Inhibition of pro-inflammatory cytokines, either by synthesis inhibitors, inhibitors of cleavage from the cell membrane, by direct antagonists, or by antibodies, reduces pain and hyperalgesia in most models studied (Urban 2000; Sommer 2001; Wijnker et al. 2004; Kopp et al. 2005).

The major pro-inflammatory cytokines that are thought to be involved in pathogenesis of neuropathic pain include interleukin-12 (IL-12), interferon-gamma (IFN-γ), and tumor necrosis factor-alpha (TNF-α) (Ji and Strichartz 2004; Vikman et al. 2003; Verri et al. 2005).

1.4.1 *IL-12*

IL-12 is produced by macrophages and dendritic cells in response to antigenic stimulation (Ehlers et al. 2005). It is a heterodimer of 70kDa that is composed of p35 and p40 subunits (Constantinescu et al. 2005). IL-12 primarily stimulates natural killer (NK) cells and naïve T cells, leading naive T cells to differentiate toward the type I T helper (Th1) cells (Pagenstecher et al. 2000). Both NK and Th1 cells subsequently produce IFN-γ and TNF-α for the development of the inflammatory response involved in nociceptive information

transmission (Marcenaro et al. 2005; Nath et al. 2005). IL-12 binds to two IL-12 receptors, β1 and β2, to exert its effect on NK and T cells (Ireland et al. 2005). Antibody against IL-12 is reportedly able to inhibit the neuroimmuno response that induced by IL-12 (Bright et al. 1998).

1.4.2 *IFN-γ*

IFN-γ is a 17-kDa polypeptide produced mainly by Th1 cells, NK cells, monocytes, macrophages, and neurons in response to the presence of foreign antigens (Yun et al. 2000; Dorries 2001; Dafny and Yang 2005; Lee et al. 2006). It can be released for extended periods of time in the CNS and peripheral nervous system (PNS) during inflammatory and infectious events, and alter synaptic activity in DRG neurons and thereby contribute to neuronal hyperexcitability (Vikman et al. 2003). IFN-γ is known to exert its biological functions by binding to two receptors, IFN-γ receptor Ⅰ (IFNGR1) and IFN-γ receptor Ⅱ (IFNGR2) (Schrijver et al. 2004), while its function can be significantly reduced by anti-IFN-γ antibodies (Shaked et al. 2005).

1.4.3 *TNF-α*

TNF-α is a 17.5 kDa, 157 amino acid protein synthesized and released by different cell types, including Th1 cells, macrophages, Schwann cells, and astrocytes following nerve injury (Monney et al. 2002; Ohtori et al. 2004; Gambino et al. 2005).

TNF-α exerts its effect through two known receptors, the TNF receptor 1 (TNFR1) and the

TNF receptor 2 (TNFR2) (George et al. 2005). Most TNF-α biological activity is elicited through the TNFR1, rather than TNFR2 (Sommer et al. 1998; Holmes et al. 2004). Following peripheral nerve injury, expression of TNFR1 has been shown to be upregulated on neuronal cells and non-neuronal cells within DRG, while TNFR2 expression occurs exclusively on non-neuronal cells (Li et al. 2004; Inglis et al. 2005).

In vitro studies show that exogenous TNF-α in DRG elicits higher and longer-lasting neuronal discharges in afferent fibers after nerve injury, indicating sensitization to endogenous TNF-α may be essential for the development and maintenance of neuropathic pain (Schafers et al. 2003). Injection of TNF-α into DRG *in vivo* induces animals to a state of allodynia, whereby non-noxious stimuli are perceived as pain. Further, if an injured DRG was injected, a significantly lower dose of additional TNF-α is needed to induce allodynia with an even faster onset (Schafers et al. 2003). Thus, both *in vivo* and *in vitro* evidence imply that injured nerve fibers are sensitized to the excitatory effects of TNF-α.

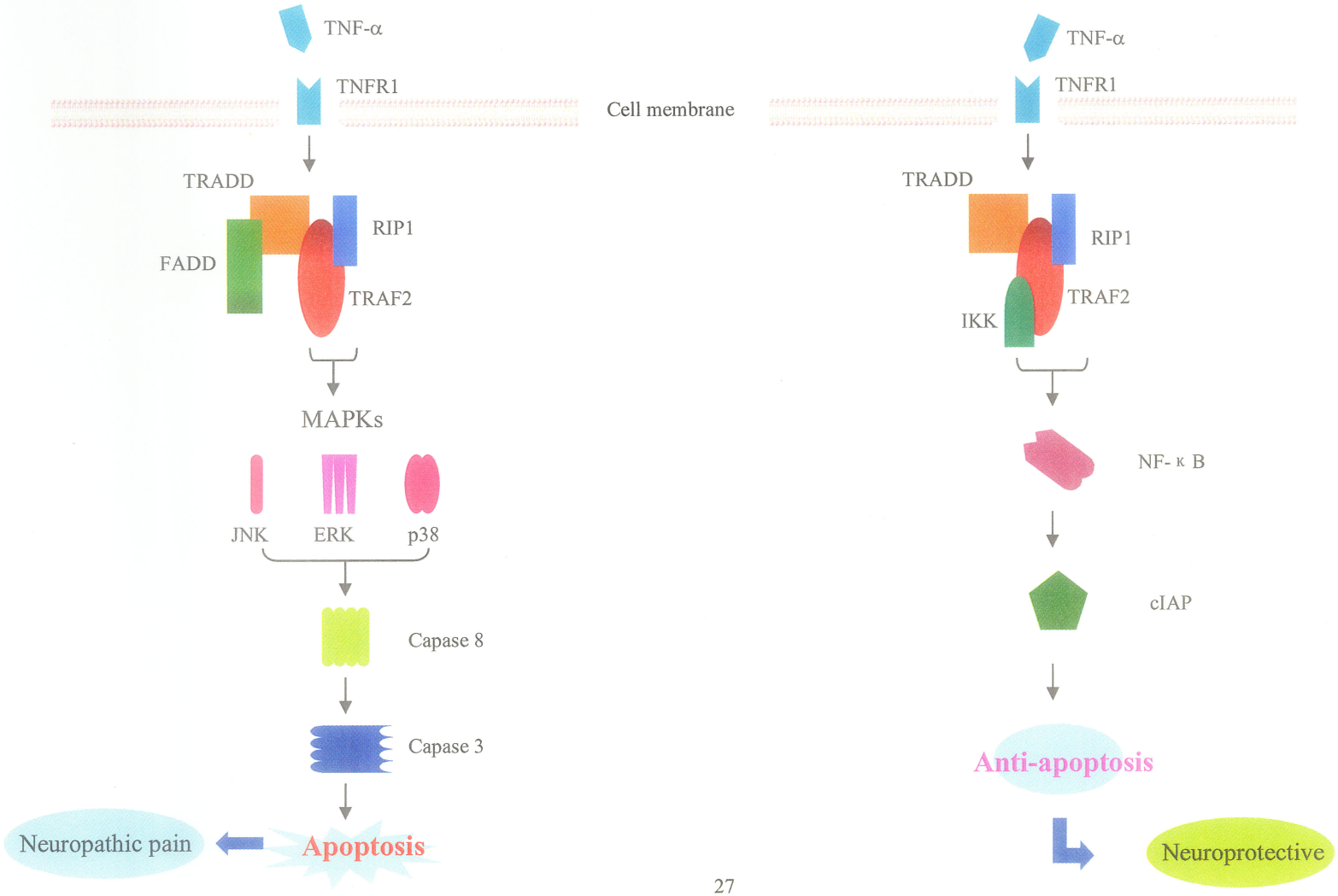
Following peripheral nerve injury, neuronal apoptosis is found to occur within the DRG (Zhou et al. 2005). Recent researches have shown that the neuronal apoptosis can be suppressed by Ca^{2+} channel antagonists (Kobayashi and Mori 1998). Moreover, Azkue *et al* reported that the sensory neuronal apoptosis can be fully blocked by MK-801, a known NMDA receptor antagonist (Azkue et al. 1998). Based on these findings, apoptosis of sensory neurons due to PNI may create a central state neuronal hyperexcitability at the level of spinal cord and thereby activate ascending pain transmission pathways which further result

in the development of chronic neuropathic pain. Although cell death is known to occur post-injury, current research suggests that TNF-α may be an integral factor involved in the injury-induced destruction/apoptosis of sensory neurons housed within the DRG (Robertson et al. 2001). Since death of sensory neurons is already linked to neuropathic pain, TNF-α appears to be a viable target for attenuation of this type of chronic pain syndrome.

Downstream effects of TNF-α in neuronal apoptosis are mediated mainly through the mitogen-activated protein kinases (MAPKs) and nuclear factor-κB (NF-κB) signaling pathways (**Figure 1**, van Vliet et al. 2005). Following peripheral nerve injury, TNF-α binds to TNFR1, and promotes association of the adaptor protein TNFR-associated death domain (TRADD), which in turn recruits other proteins such as Fas-associated death domain protein (FADD), the adaptor protein TNFR-associated factor 2 (TRAF2), and the serine and threonine protein kinase receptor-interacting protein type 1 (RIP1). All three MAPK subgroups, the extracellular signal–regulated kinases (ERKs), the stress-activated c-Jun N-terminal kinases (JNKs), and the p38 kinases are then activated by TRAF2 and RIP1 (Devin et al. 2003). The activated MAPKs are efficient inducers of caspase-8, which subsequently leads to activation of caspase-3 that can cleave multiple cellular proteins, resulting in apoptosis of neuronal cells (Baud and Karin 2001).

Conversely, at normal physiological concentrations TNF-α may exert neuroprotective functions within the designated tissues by triggering NF-κB pathway. At normal physiological conditions, the interaction of TNF-α and TNFR1 initiates activation of

Figure 1: TNF-α signaling pathways in neuronal apoptosis



TRADD, TRAF2, and RIP1. Both TRAF2 and RIP1 are involved in the activation of NF-κB by recruiting the inhibitor of κB kinase (IKK) (Baud and Karin 2001). NF-κB results in anti-apoptotic response through the induction of cellular inhibitors of apoptosis (cIAPs), which function as specific caspase inhibitors (Deveraux and Reed 1999).

Increasing evidence implicates dysregulation of TNF-α expression or signaling in the pathology of many diseases, including rheumatoid arthritis, Crohn's disease, and MS (Balkwill et al. 2000). Three TNF-α antagonists, adalimumab (Abbott), infliximab (Schering-Plough), and etanercept (Amgen), are now licensed by the FDA for clinical use in rheumatoid arthritis and Crohn's disease (Nestorov 2005). Adalimumab and infliximab are human anti-TNF-α monoclonal antibodies, while etanercept is a soluble dimeric fusion protein containing the ligand-binding domain of TNFR2. In consistent with that monotherapy with antibody against TNF-α reduces hyperalgesia in animal models of neuropathic pain (Schafers et al. 2001; Sommer et al. 2001), preliminary clinical studies suggest that TNF-α antibodies, such as etanercept, are also useful for providing pain relief in patients with rheumatoid arthritis and cancer-induced neuropathic pain (Tobinick 2003; Flagg et al. 2005). In addition, infliximab has been found to reduce temporomandibular joint pain in rheumatoid arthritis (Kopp et al. 2005). Furthermore, there are three drugs have been approved in immunomodulatory treatment for relapsing-remitting MS (RRMS), including Avonex (IFN-β1a, Biogen), Rebif (IFN-β1a, Serono), and Betaseron (IFN-β1b, Berlex) (Vartanian et al. 2004). These three drugs inhibit Th1 activities and subsequently suppress the functions of pro-inflammatory cytokines, such as IL-12, IFN-γ, and TNF-α (Sega et al. 2004). They all

reportedly reduce the frequency of attacks and the progression of chronic pain syndromes in RRMS (Ryan and Piascik 2002).

Based on the above background information, the DRG is known to have a critical role in the development of neuropathic pain. Although the exact underlying etiology of neuropathic pain is still unknown, death of sensory neurons within the DRG leads to its development (Zimmermann 2001). Death of sensory neurons can occur via drug, disease, or injury-induced causes (Fischer et al. 2001; Leinninger et al. 2004; Kuo et al. 2005). Specifically, peripheral nerve injuries, such as constriction, crush, ligation, and axotomy, interrupt nutritive support obtained for DRG sensory neurons from their respective peripheral targets (skin, muscle, joints, etc.), and subsequently lead to neuronal death (Zimmermann 2001; Dowdall et al. 2005). In addition, following such nerve injuries, inflammatory cytokines such as TNF-α are known to be involved in the development of neuropathic pain through its downstream cell signaling pathways by activating capase 8 and capase 3 that eventually result in neuronal apoptosis (Baud and Karin 2001; Ji and Strichartz 2004). Although TNF-α has also been known to exert beneficial functions at normal physiological concentrations (Deveraux and Reed 1999), it is assumed that peripheral nerve injury may trigger an abnormal burst of TNF-α that facilitates the early developmental cascade of neuropathic pain (Ji and Strichartz 2004). Henceforth, our current research has focused on determining the effect that peripheral nerve axotomy has on TNF-α expression within the DRG, so that we can better understand their involvement in underlying pathogenesis of neuropathic pain, and thereby provide new avenues for possible therapeutic intervention.

2. HYPOTHESIS

Sciatic nerve axotomy induces the upregulation of TNF-α within the DRG

3. AIMS

3.1 To determine if TNF-α is upregulated within the DRG following sciatic nerve axotomy.

3.2 To determine the cellular source of TNF-α within the DRG

4. MATERIALS AND METHODS

A variety of experimental animal models of neuropathic pain have been developed in the past decade (Eaton 2003; Gabay and Tal 2004; Erichsen et al. 2005). The most common used animal models include: the chronic constriction injury, the spinal nerve ligation, the partial sciatic nerve ligation, the tibial and sural transaction, and the complete sciatic nerve axotomy (Wall et al. 1979; Bennett and Xie 1988; Seltzer et al. 1990; Kim and Chung 1992; Lee et al. 2000; Dowdall et al. 2005). In order to obtain robust results, here we deicided to use sciatic nerve axotomy model which is able to completely disconnect the DRG from its peripheral neurotrophic sources.

Juvenile, male and female Sprague-Dawley rats (11 days old, 30-35g body weight) were purchased from Central Animal Breeding Facility of the University of Manitoba, and maintained under temperature controlled conditions (20°C) of an artificial 12 hour light/dark cycles with food and water *ad libitum*. In conducting the research described in this report, all animals received humane care in compliance with the guidelines of the animal care and use

committee of the University of Manitoba (Animal Protocol number: F02-020), which is in accordance to the Canadian Council on Animal Care criteria. A total of 24 rats were randomly divided into three experimental groups: naïve control (n=6), sham (n=6), and peripheral nerve lesion (PNI, n=12). The PNI group underwent unilateral axotomy of sciatic nerve after being anesthetized with inhaled isoflurane (Pharmaceutical Partners of Canada, Richmond Hill, ON). The transected sciatic nerve was ligated to prevent reinnervation. The sham group underwent surgery to only expose the sciatic nerve without manipulation or touching. The naïve control group had no surgical manipulation. Animals were sacrificed at day 8 or 15 (**Table 1**). Both ipsilateral and contralateral DRG were harvested for immunohistochemical and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. At the day of tissue harvesting, half amount of the animals underwent cardiac-perfusion-fixation. The DRG obtained from these animals were designated for immunohistochemical analysis. The DRG obtained from the remaining animals were rapidly collected without fixation for RT-PCR purposes (**Figure 2**).

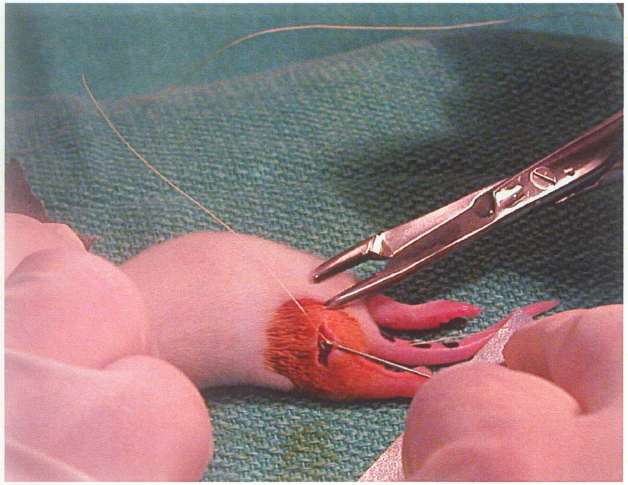
Table 1: Flow chart of tissue harvesting

	Naïve control	Sham	PNI	
Number of Animals	6	6	6	6
Sacrificed date	day 8	day 8	day 8	day 15

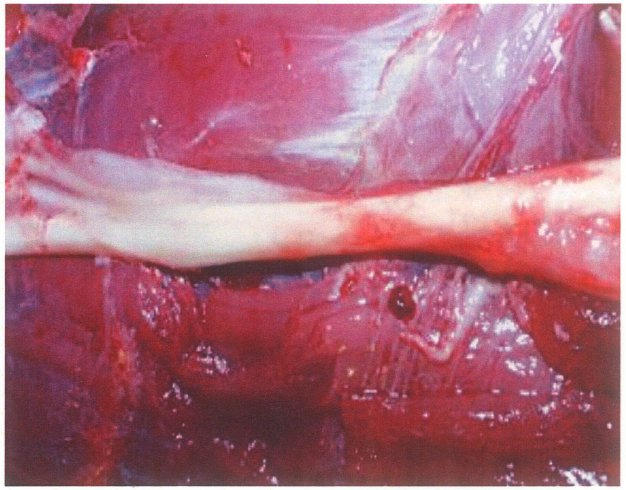
4.1 *Immunohistochemistry*

Double-immunofluorescence staining was conducted according to previously established methods in order to localize TNF-α expression within the DRG (Namaka et al. 2001)

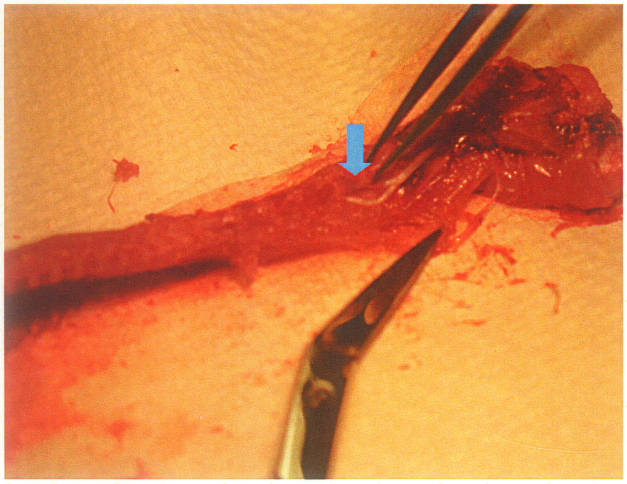
Figure 2: Surgeries



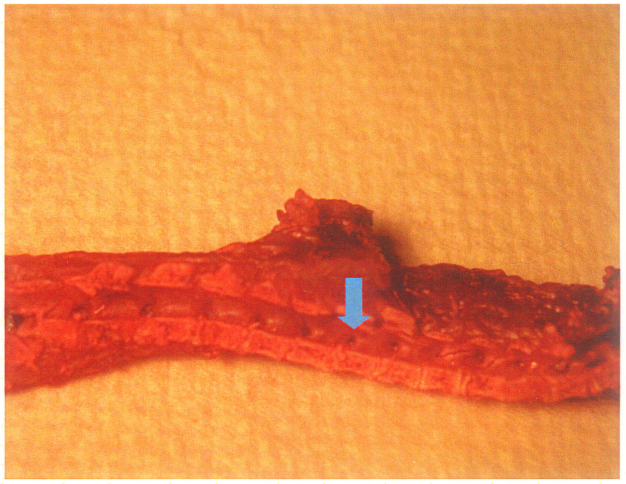
1. Unilateral sciatic nerve axotomy



2. Exposure of sciatic nerve without transection



3. DRG harvesting, the arrow indicates sciatic nerve



4. Vertebral column following removal of DRG, the arrow indicates the hole that DRG is hidden in.

4.1.1 *Preparation of pre-fix solution*

To prepare 100ml pre-fix solution, 0.9g NaCl (Sigma, St. Louis, MO), 0.1g $NaNO_3$ (Fisher, Fair Lawn, NJ), and 100 IU heparin (Leo, Denmark) were added into 80ml distilled water, and stirred to dissolve, then *qs* to 100ml with distilled water.

4.1.2 *Preparation of fixative solution*

4.1.2.1 *Preparation of 0.5M phosphate stock solution*

To make 1 liter 0.5M phosphate stock solution, 70.97g of sodium phosphate dibasic anhydrous (Na_2HPO_4, MW=141.96, Sigma, St. Louis, MO) was added into 900ml of distilled water, and warmed to dissolve well, then cooled to room temperature, *qs* to 1000ml (solution #1). While the initial solution was cooling, a second solution (#2) was prepared by dissolving 17.33g of sodium phosphate monobasic monohydrate ($NaH_2PO_4 \cdot H_2O$, MW=137.99, Sigma, St. Louis, MO) in 200ml of distilled water, and *qs* to 250ml. The solution #1 was then added to solution #2 until a pH of 7.4 was achieved. The resultant solution mixture was then stored at room temperature.

4.1.2.2 *Preparation of fixative solution*

To make 100ml fixative solution, 4g paraformaldehyde (Fisher, Fair Lawn, NJ) was added into 50ml distilled water, and heated to 65°C while stirring. The solution was then cleared with 1-2 drops of 10N NaOH while cooling to room temperature. 20ml 0.5M phosphate buffer solution was then added, and filtered with #1 filter paper (Whatman, Maidstone, England). pH was adjusted to 7.0 with 1N HCl, and *qs* to 100ml with distilled water.

4.1.3 *Preparation of post-fix solution*

To make 100ml post-fix solution, 10g sucrose (Boehringer Mannhem, Hawthorne, NY) and 0.1g sodium azide (Mallinckrodt, Paris, KY) were added into 80ml fixative solution, and stirred to dissolve, *qs* to 100ml with fixative solution.

4.1.4 *Preparation of phosphate buffer saline (PBS)*

To make 1 liter PBS solution, 9g NaCl was dissolved in 100ml distilled water. 200ml 0.5M phosphate buffer stock solution was then added, *qs* to 1000ml. The solution was filtered with 0.22μm membrane (Whatman, Maidstone, England), and stored in autoclaved bottle at 4°C.

4.1.5 *Preparation of PBS-Triton (PBS-T) solution*

To make 100ml PBS-T solution, 0.3ml Triton X-100 (Sigma, St. Louis, MO) was gently added into 100ml PBS solution, and stirred to mix well.

4.1.6 *Preparation of 50mM Tris-HCl solution*

4.1.6.1 *Preparation of 1M Tris-HCl stock solution*

To make 500ml 1M Tris-HCl stock solution, 60.55g Trizma base (Sigma, St. Louis, MO) was added into 200ml distilled water and stirred to dissolve well, 6N HCl was then slowly added until pH 7.4 was achieved, be careful when adding HCl close to pH 7.4 because it may jump up quickly at that point. *qs* to 500ml with distilled water, and stored at room temperature.

4.1.6.2 *Preparation of 50mM Tris-HCl solution*

To make 100ml 50mM Tris-HCl solution, 5ml 1M Tris-HCl stock solution was added to 95ml distilled water, and stirred to mix well.

4.1.7 *Immunohistochemical staining*

Half amount of the animals designated for immunohistochemical analysis underwent cardiac-perfusion-fixation using perfusion pump (Cole-Parmer Instrument, Anjou, QC) with pre-fix followed by fixative solution. DRG were then surgically extracted and immersion fixated in fixative solution for another 1 hour. The harvested tissue was subsequently stored in post-fix solution at 4°C. The L4 DRG was then cryostat sectioned at a 10μm thickness and mounted on frosted slides (Fisher, Fair Lawn, NJ), stored at -20°C. Immunofluorescent analysis of DRG cryostat sections was conducted to detect the expression of TNF-α as previously described (Murata et al. 2004). In brief, the slides were washed with PBS-T three times by 20 minutes intervals, and incubated with the mixture of primary antibody against TNF-α (1:100, R & D system, Minneapolis, MN), primary antibody against Neun (1:1000, Chemicon, Temecula, CA), and sheep serum (1:100, Sigma, St. Louis, MO) in 4°C for two days. The primary antibody against TNF-α localized TNF-α expression, while Neun labeled the nuclei of the neurons (Collombet et al. 2006). Following incubation with primary antibodies, the slides were washed with PBS-T three times, and incubated with the mixture of secondary antibodies (donkey anti-goat IgG, 1:100, Molecular Probes, Eugene, OR; goat anti-mouse FITC, 1:50, Jackson, West Grove, PA) and sheep serum (1:100) at room temperature for 1.5 hours. As a result, TNF-α was visualized as red, while the nuclei of the

sensory neurons were visualized as green under fluorescent microscope. The slides were then washed with PBS-T once and 50mM Tris-HCl twice. Following the removal of Tris-HCl, one drop of mounting medium (Vector Labs, Burlingame, CA) was added to the tissue which was subsequently sealed with a coverslip using nail polish. The slides were stored at -20°C. An Olympus BX51 fluorescent microscope and Alexa 568 fluorescent cube (Olympus, Japan) was employed to visualize the double staining. The picture processing and cell measurements were performed using the software *Image Pro Express* (Media Cybernetics). The quantification of TNF-α positive neurons from each respective section of DRG tissue was identified based on the definitions of TNF-α positive and negative neurons illustrated in **Figure 6**.

4.2 *RT-PCR*

The other half amount of animals designated for RT-PCR analysis were sacrificed by decapitation following a lethal dose of ketamine (20mg/100g body weight, Bimeda-MTC, Cambridge, ON) and xylazine (2mg/100g body weight, Bayer, Shawnee, KS). The DRG were rapidly collected, gathered and frozen in dry ice (Praxair, Winnipeg, MB), and removed to -70°C within 60 minutes. RT-PCR was conducted according to previously established methods in order to determine TNF-α expression within the DRG (Shen et al. 2003).

4.2.1 *Total RNA isolation*

Tissue samples were homogenized in 1 ml Trizol Reagent (Invitrogen, Carlsbad, CA) using a power homogenizer (Brinkmann, Switzerland). The homogenized samples were then

incubated at room temperature for 5 minutes to permit the complete dissociation of nucleoprotein complexes followed by the addition of 0.2 ml of chloroform (Fisher, Fair Lawn, NJ). Sample tube caps were securely fastened to prevent leakage and sample loss. The sample tubes were then shaken vigorously by hand for 15 seconds and incubated at room temperature for 2 to 3 minutes. The samples were then centrifuged at 12,000*g* for 15 minutes at 4°C by using a microcentrifuge (International Equipment, Needham heights, MA). Following centrifugation, the mixture separates into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase which is about 60% of the volume. The aqueous phase was extracted and transferred to a fresh tube where the total RNA was precipitated by mixing with 0.5 ml isopropyl alcohol (Sigma, St. Louis, MO). The samples were then incubated at room temperature for 10 minutes and centrifuged at 12,000*g* for 10 minutes at 4°C. The RNA precipitate, which was invisible before centrifugation, forms a gel-like pellet on the side and bottom of the tube. The supernatant was removed, and the RNA pellet was washed once with 1 ml of 75% ethanol (Fisher, Fair Lawn, NJ). The sample was mixed by vortexing, and centrifuge at 7,500*g* for 5 minutes at 4°C. The resultant RNA pellet was then air-dried for 10 minutes. Following this, the RNA was dissolved in RNase-free water by passing the solution a few times through a pipette tip, and incubating for 10 minutes at 60°C. At the end of the procedure, the concentration of total RNA was determined using ultraviolet (UV) spectrometer (Smart spectrum 3000, Bio-rad, Hercules, CA). The RNA was then quantitatively diluted to a concentration of 1 μg/μl with RNase-free water, and subsequently stored in -70°C.

4.2.2 *Reverse transcriptase (RT)*

After thawing the total RNA stock solution on ice, to each of 1μg RNA (1μl), added 1μl oligo$(dT)_{12-18}$ primers (Beckdon Dickinson, Palo Alto, CA), 1μl 10mM deoxynucleoside triphosphate (dNTP) mix (Invitrogen, Carlsbad, CA), and RNase-free water to 12μl. The resultant mixture was then heated at 65°C for 5 minutes and quickly chilled on ice. The contents of the tube were then collected by brief centrifugation. Added 4μl 5× First-strand buffer (Invitrogen, Carlsbad, CA), 2μl 0.1M dithiothreitol (DTT, Invitrogen, Carlsbad, CA), and 1μl RNaseOUT recombinant ribonuclease inhibitor (40units/μl, Invitrogen, Carlsbad, CA), then mixed the contents of the tube gently by a pipette tip and incubate at 37°C for 2 minutes. Added 1μl moloney-murine leukemia virus reverse transcriptase (M-MLV RT, Invitrogen, Carlsbad, CA), and mixed by pipetting. Incubated at 37°C for 50 minutes, and then inactivated the reaction by heating at 70°C for 15 minutes. At the end, 80μl RNase-free water was added into the tube to make the total volume 100μl. The cDNA was then ready to be used as a template for polymerase chain reaction (PCR) amplification.

4.2.3 *PCR*

4.2.3.1 *Design of primers*

In PCR amplification, a pair of small pieces of synthetic DNA, that are complementary of the target sequence, serve as primers. The primers were designed based on the target sequence published at Genbank (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide), using a software called *Oligo 5.0* (Cambio). At the search engine of Genbank, find the corresponding sequence and copy. Open Oligo 5.0, click 'File/New', click 'Edit/Paste' to

paste the sequence into Oligo. Click 'Accept/Discard/Accept'. Click 'Search/for primers and probes'. At the window of 'Search/for primers and probes', check all the items except 'compatible with the upper primer', 'compatible with the lower primer', 'sequencing primers', 'hybridization probes', and 'continue false priming search in other file(s)'. Keep search mode at 'mark'. Click 'parameters' at the window of 'search for primers and probes'. At the window of 'search parameters', set search stringency at 'very high' and 'automatically change stringency'. Check 'adjust length to match Tm's', uncheck 'inverse PCR', and set the parameters as following:

Oligonucleotide length: 20 nt

Acceptable 3'-dimer delta G: -2.0kcal/mol

Maximum length of acceptable dimers: 2 base pairs

3'-terminal nucleotides checked for dimers: 20

3'-terminal stability range: -6.5 to -9.0kcal/mol

GC clamp stability: -11.0kcal/mol

Minimum acceptable loop delta G: 0cal/mol

Oligo Tm range [45.5 to 82.6]: 54.8 to 73.3°C

Max acceptable false priming efficiency: 150 points

At the window of 'search parameters', click 'search ranges'. At the window of 'search ranges', set 'PCR product length' at 150-400, and leave the other parameters as they are. Click 'OK' for both windows, the system will perform primer search. At the window of 'search status', hit 'primer pairs'. All the possible primers that match the settings are listed. Highlight either one of the primers, open up analyzing windows by clicking 'Analyze/Duplex

Formation/Upper Primer', 'Analyze/Duplex Formation /Lower Primer', and 'Analyze/Duplex Formation/Upper/Lower'. Choose different primers on the list while keeping the three analyzing windows and the window of 'PCR' open. The applicable primers are selected according to following criteria: 17-22 bases in length, the percentage of G+C is less than 60%, annealing temperature between 50-70°C. Three or more Cs or Gs at the 3' end of primers, self-complementarity (hairpin structure), and complementarity btween the two primers at 3' end should be avoided.

4.2.3.2 *Preparation of Tris-borate-EDTA (TBE) buffer*

To prepare 1 liter TBE buffer, 108g Tris base (Sigma, St. Louis, MO), 55g boric acid (Sigma, St. Louis, MO), and 9.8g $Na_2EDTA \cdot 2H_2O$ (Sigma, St. Louis, MO) were added to 1 liter water. The solution was heated to dissolve, and stored at room temperature.

4.2.3.3 *Preparation of loading buffer*

To prepare 100ml loading buffer, 0.2ml 12.5% bromphenol (Sigma, St. Louis, MO), 0.2ml 12.5% xylene cyanole (Sigma, St. Louis, MO), and 25g Ficoll 400 (Sigma, St. Louis, MO) were dissolved in 100ml water. The solution was filtered through 0.4μm membrane, and stored at 4°C.

4.2.3.4 *Preparation of Agarose gel*

0.7g Agarose (Sigma, St. Louis, MO) was weighed in a bottle. 70ml 1% TBE buffer was then added. The solution was heated for 1:20 in microwave with the cap of the bottle loosen. A

volume of ethidium bromide solution that equivalent to 35μg ethidium bromide (Sigma, St. Louis, MO) was added. The resultant solution was then poured into electrophoresis tray, and allowed to cool down for 1 hour with the comb on.

4.2.3.5 *Amplification*

In order to ensure the tissues are processed correctly, the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a housekeeping gene found in all tissues at high level, needed to be detected prior to analyzing the expression of TNF-α. Added the following to a PCR reaction tube for a final reaction volume of 50μl, using water as negative control and rat spleen as positive control:

0.5μl *Taq* DNA polymerase (Qiagen, Hilden, Germany)

10μl Q buffer (Qiagen, Hilden, Germany)

5μl PCR 10× buffer (Qiagen, Hilden, Germany)

4μl 50 mM $MgCl_2$ (Qiagen, Hilden, Germany)

1μl 10 mM dNTP (Invitrogen, Carlsbad, CA)

1μl upper primer for GAPDH (**Table 2**, Invitrogen, Carlsbad, CA)

1μl lower primer for GAPDH (**Table 2**, Invitrogen, Carlsbad, CA)

22.5μl RNase-free water

5μl cDNA (from the RT reaction)

Table 2: sequence of GAPDH primers (Genbank number: NM017008):

	Sequence	Temperature	length
upper	5' TTC TTG TGC AGT GCC AGC CTC GTC 3'	60°C	203bp
lower	5' GCC GTT GAA CTT GCC GTG GGT AGA 3'		

Gently mixed the contents and mount the tubes to PCR thermal cycler (Mastercycler, eppendorf, Westbury, NY) for amplification. Since 38 cycles were required for the amplification, the temperature program was set as following:

1. 95°C 5 minutes
2. 95°C 1 minute
3. 60°C 0.5 minutes
4. 72°C 1.5 minutes

repeat the step 2 to step 4 for another 37 cycles

5. 72°C 8 minutes
6. Hold at 4°C

Following the amplification finished, the tubes were briefly centrifuged. 10μl PCR products (double-stranded DNA) and 2μl loading buffer were gently mixed on wax paper, and loaded onto 1% agarose gel that contains 0.5μg/ml ethidium bromide. Electrophoresis was run in TBE buffer for 15 minutes. The agarose gel was viewed under UV light. Photograph was taken if necessary. Only if the luminosity of the GAPDH bands looked very similar, the TNF-α assay can be started.

To determine the expression of TNF-α, followed the same procedure but replace the primers for GAPDH by primers for TNF-α (**Table 3**, Invitrogen, Carlsbad, CA). Since 32 cycles of the PCR amplification were required, the temperature program was set as following:

1. 95°C 5 minutes
2. 95°C 1 minutes
3. 59°C 0.5 minutes
4. 72°C 1.5 minutes

repeat the step 2 to step 4 for another 31 cycles

5. 72°C 8 minutes
6. Hold at 4°C

Table 3: sequence of TNF-α primers (Genbank number: AF329982)

	Sequence	Temperature	length
upper	5' AGC CGA TTT GCC ATT TCA TAC CAG 3'	59°C	247 bp
lower	5' CAC GCC AGT CGC TTC ACA GAG 3'		

The photographs were taken with instant camera (DS34, Polaroid), then scaned and electronically saved. The luminosity of the electrophoresis bands was analyzed by *ImageJ* 1.34 (Shareware, can be downloaded at http://rsb.info.nih.gov/ij/ download.html).

4.2.3.6 *Data analysis using ImageJ*

Open *ImageJ*, import the picture by clicking 'File/Open'. The picture should be shown as

white bands on black background. Select all the bands using 'Rectangular Selections' tool. Hit 'Analyze/Gels/Select first lane', and then click 'Analyze/Gels/Plot lanes'. The luminosity of the bands is converted to curve. Draw a straight line along the bottom of the peaks using 'straight line selections' to make each peak a closed area. Click 'Wand (tracing) tool', select every individual peak one by one. The value of peak area is shown in a new window. Graph is made based on the value of area using Excel. In brief, ImageJ converts the luminosity of the bands to a curve. The area under the curve quantifies the relative luminosity of the bands. Expression of TNF-α was then normalized by comparing the area under the curve of TNF-α as a ratio to that of endogenous GAPDH.

4.3 *Real-time PCR*

In order to fully quantify TNF-α expression within the DRG, Real-time PCR was performed using Real-time PCR thermal cycler (Lightcycler, Roche diagnostics, Mannheim, Germany), with QIAquick PCR purification kit (Qiagen, Hilden, Germany) and Lightcycler FastStart DNA Master SYBR Green I kit (Roche diagnostics, Mannheim, Germany).

4.3.1 *Purification of double-stranded DNA*

According to QIAquick PCR purification kit (Qiagen, Hilden, Germany) manufacturer's instruction, 5 volumes of buffer PB was added to 1 volume of the TNF-α PCR products. A QIAquick spin column was placed in a provided 2ml collection tube. The sample was applied to the QIAquick column and centrifuged for 30 seconds. The flow-through was discarded. The QIAquick column was then placed back into the same collection tube. 0.75ml buffer PE

was added to the QIAquick column and centrifuged for 30 seconds. The flow-through was discarded again. The QIAquick column was placeed back in the same collection tube. The column was centrifuged for an additional 1 minute. The QIAquick column was then placed in a clean 1.5ml microcentrifuge tube. To elute DNA, 25μl buffer EB was added to the center of the QIAquick membrane and centrifuged the column for 1 minute, this step was done twice to ensure complete elution of bound DNA. The purified double-stranded DNA of TNF-α was collected in the 1.5ml microcentrifuge tube.

4.3.2 *Standard curves*

Real-time PCR thermal cycler is programmed to determine the fluorescence of the reaction at each cycle of the amplification, which is able to fully quantify the double-stranded DNA concentrations by making cycle dependent curves. Standard curves represent known concentrations. The purpose of standard curves is to compare the known concentrations with the unknown concentration of each tested sample. In detail, the concentration of purified double-stranded DNA was measured three times using UV spectrometer, and the average was calculated. Following equations were applied to make different concentrations of double-stranded DNA:

Known molecular weight of 1 base pair (bp) of double-stranded DNA=660Da (Roche Applied Science Lab FAQs, 2nd edition, Roche Diagnostics, Penzberg, Germany, 2002).

Known Avogadro's number: $1g=6.023\times10^{23}$ Da, $1Da=1.660\times10^{-24}$ g

Therefore, 1 bp=$660Da\times1.660\times10^{-24}$ g/Da=1.096×10^{-21} g

Hence, 1 copy of double-stranded DNA=length$\times 1.096\times 10^{-21}$ g/bp

1 copy of TNF-α double-stranded DNA=247 bp$\times 1.096\times 10^{-21}$ g/bp=2.707×10^{-19} g

Since the average concentration obtained from UV spectrum of TNF-α double- stranded DNA is 112.79μg/ml=112.79×10^{-9} g/μl.

Therefore, the concentration of TNF-α double-stranded DNA in copies is:

$$\frac{112.79\times 10^{-9}\ \text{g/μl}}{2.707\times 10^{-19}\ \text{g/copy}} = 4.17\times 10^{11}\ \text{copies/μl}$$

Take 2.40μl (10^{12} copies) above solution, add 97.60μl PCR grade water and mix, to make 10^{10} copies/μl stock.

Take 1μl 10^{10} copies/μl, add 99μl PCR grade water and mix, to make 10^{8} copies/μl.

Take 1μl 10^{8} copies/μl, add 99μl PCR grade water and mix, to make 10^{6} copies/μl.

Take 1μl 10^{6} copies/μl, add 99μl PCR grade water and mix, to make 10^{4} copies/μl.

Use PCR grade water as 0copy/μl.

According to manufacturer's instruction of Lightcycler FastStart DNA Master SYBR Green I kit (Roche diagnostics, Mannheim, Germany), the "Hot Start" reaction mix was prepared as described following. One vial 1a containing the Lightcycler FastStart Enzyme and one vial 1b containing the Lightcycler FastStart Reaction Mix SYBR Green I were briefly centrifuged. A total volume of 10μl was trnsfered from vial 1a into vial 1b. The resultant solution was gently mixed by pipette. Vial 1b was relabled with the new labels provided with the kit (vial 1: Lightcycler FastStart DNA Master SYBR Green I), one for the top of the cap and one for the side of the vial. The vial was protected from light by wrapping with aluminum foil.

To obtain TNF-α standard curve of Real-Time PCR, the following were added to a microcentrifuge tube for a final reaction volume of 20μl:

9.4μl PCR grade water

1.6μl $MgCl_2$

1μl upper primer for TNF-α

1μl lower primer for TNF-α

2μl SYBR mix (vial 1)

5μl double-stranded DNA (10^8, 10^6, 10^4, 0 copies/μl, respectively)

The contents of each tube were mixed gently by pipette and transfered to a Lightcycler capillary (Roche diagnostics, Mannheim, Germany). The Lightcycler capillaries were briefly centrifuged to ensure the contents stay at the bottom end of the capillaries without any air bubble. The capillaries were then mounted into Real-time PCR thermal cycler. The temperature parameters at the computer that connects to the thermal cycler were set according to manufacturer's instruction (See appendix #1 for detail), then run the Real-time PCR thermal cycler. The data of standard curves (**Figure 3**) was saved in the computer connecting to the cycler. The black, red, green, and blue curve represents 10^8, 10^6, 10^4, and 0 copies/μl double-stranded DNA, respectively.

4.3.3 *Amplification of samples*

The following were added to a microcentrifuge tube for a final reaction volume of 20μl:

9.4μl PCR grade water

1.6μl $MgCl_2$

1μl upper primer for TNF-α

1μl lower primer for TNF-α

2μl SYBR mix (vial 1)

5μl cDNA (RT products) of the samples

The contents of each tube were mixed, and carefully transfered to a Lightcycler capillary. The capillaries were then briefly centrifuged, and mounted into Real-time PCR thermal cycler with 10^8, 10^6, 10^4, and 0 copies/μl standard curve stocks. The temperature parameters were set same as those of standard curve. Run the Real-time PCR thermal cycler. The data of tested samples was saved in the computer.

5. RESULTS

5.1 *Immunohistochemistry*

Photographs were taken at 10×, 20×, and 40× (**Figure 4**). Consistent higher level of TNF-α was found in both ipsi- and contralateral DRG of PNI animals that euthanized at day 8. TNF-α fluorescence was consistently lower at naïve control, sham groups, and animals of active lesion, euthanized at day 15. In addition, it appeared that the neuronal cells predominantly contribute to TNF-α expression compare to the non-neuronal cells within the DRG obtained from nerve-injured animals. Furthermore, by counting the TNF-α positive and negative neurons in the photographs of 20×, the percentage of TNF-α positive neurons were depicted in **Figure 6**. The percentage of TNF-α positive neurons in the both ipsi- and

contralateral DRG of PNI animals that euthanized at day 8 (66%, 73%) was markedly higher than other groups (11%-49%, **Figure 6**). Moreover, by measuring the diameter of the neurons, size of TNF-α positive neurons of every individual group was obtained (See appendix #2 for raw data). The mean diameter of the TNF-α positive neurons in the bilateral DRG of PNI animals that were euthanized at day 8 was 23.9μm, which was significantly smaller than that of uninjured animals (naïve and sham groups, average=29.0μm) ($p<0.05$, **Figure 5**). The result suggested that these smaller diameter neurons may correspond to Aδ and C fibers that are responsible for pain transmission.

5.2 *RT-PCR*

The eclectrophoretic bands of TNF-α and GAPDH were shown on **Figure 7**. By comparing the luminosity of the TNF-α bands as a ratio to those of GAPDH, the results showed that TNF-α was upregulated in both ipsilateral and contralateral DRG of PNI, euthanized at day 8 animals, and diminished to normal level at day 15 (**Figure 8**).

5.3 *Real-time PCR*

Real-time PCR results were shown on **Figure 9** and **Figure 10**, and then analyzed by one way ANOVA using statistical software SAS (SAS Institute Inc., Cary, NC, **Table 4**). The TNF-α expression of both ipsi- and contralateral DRG for PNI day 8 animals was significantly higher than other groups ($p<0.05$, n=6). There was no statistical different between these two ($p>0.05$, n=6). There was no significant difference between sham and naïve control, neither between sham and PNI day 15 ($p>0.05$, n=6) (**Figure 11**). The Real-time PCR results were consistent with the immunohistochemical and RT-PCR results.

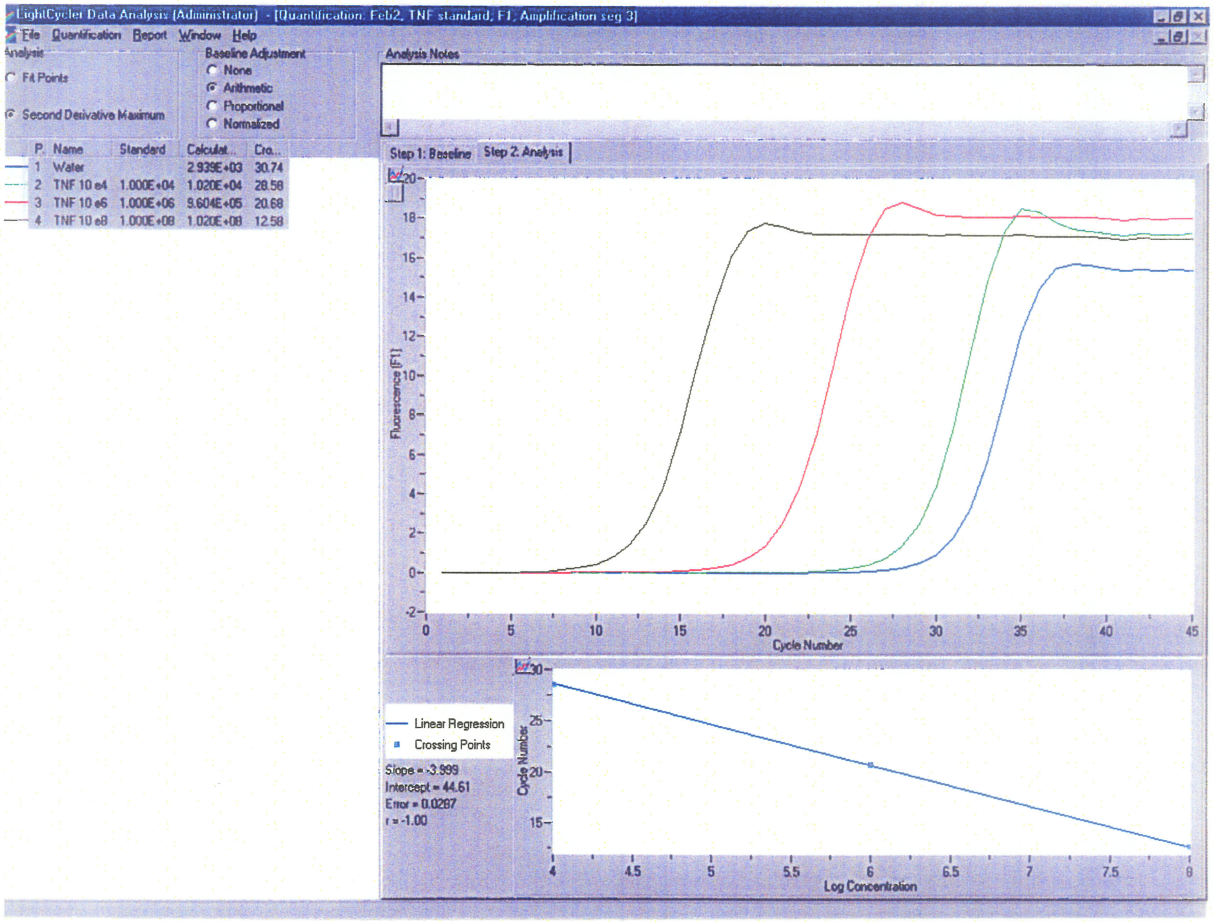


Figure 3 : Real-time PCR standard curves of TNF-α expression. The black, red, green, and blue curve represents 10^8, 10^6, 10^4, and 0 copies/ μ l double strand DNA, respectively.

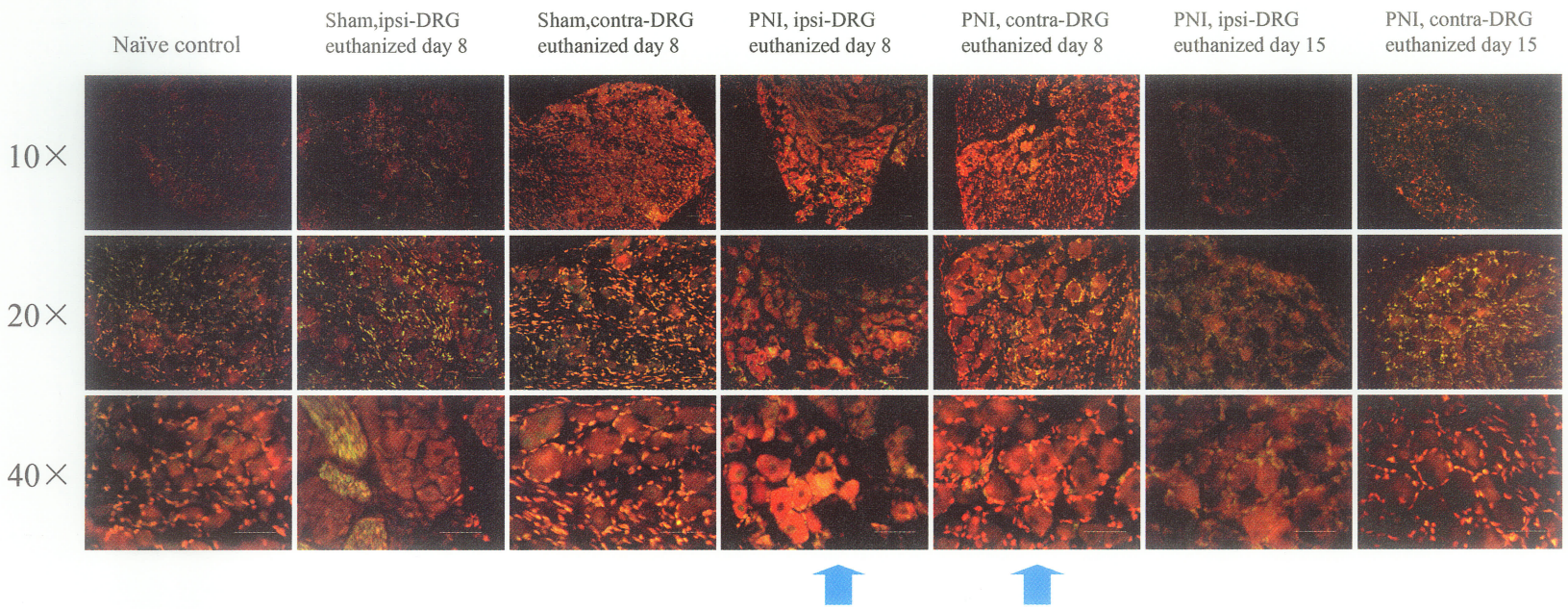
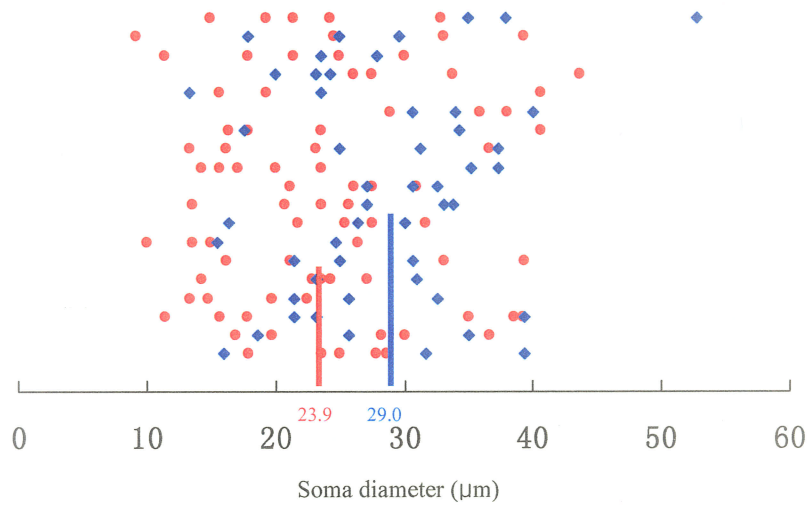


Figure 4: Immunohistochemical results of TNF-α expression within the DRG (scale bar=50μm)

1) Double staining with antibodies against TNF-α (red) and Neun (green). Constant higher TNF-α expression was found in the DRG of both ipsi- and contralateral of PNI animals that euthanized at day 8 (blue arrows).
2) There is a consistent switch in TNF-α expression from predominant satellite cell baseline source in uninjured animals to a source of predominant small to medium sized neurons with satellite cell background.
3) The size of TNF-α positive neurons in the DRG of both ipsi- and contralateral of PNI animals that euthanized at day 8 is smaller than naïve and sham groups ($p<0.05$).

Figure 5: Size of TNF-α positive neurons following axotomy (*see appendix#2 for raw data*)



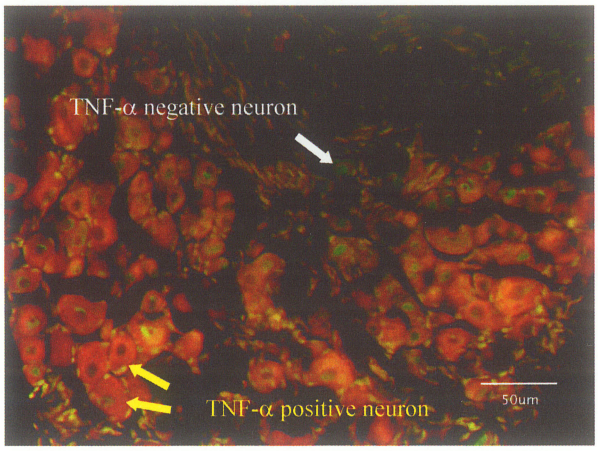
● Group 1: PNI, bilateral-DRG, euthanized day 8 (injured neurons)

◆ Group 2: Naïve control, Sham bilateral-DRG (uninjured neurons)

	Mean	Standard deviation	*p*
Group1	23.9	8.2	<0.05
Group2	29.0	8.1	

The mean diameter of the TNF-α positive neurons in the injured neurons is 23.9μm, which is significantly lower than that of uninjured neurons (mean=29.0μm, $p<0.05$)

Figure 6: Percentage of TNF-α positive neurons



The neurons that have intense TNF-α signal were defined as positive (yellow arrows), while the neurons have weak TNF-α signal were defined as negative (white arrow)

$$\frac{\text{Number of TNF-}\alpha\text{ positive neurons}}{\text{Number of total neurons}} \times 100\% = \text{Percentage of TNF-}\alpha\text{ positive neurons}$$

1. Naïve control 8/50×100%=16%
2. Sham, ipsilateral-DRG, euthanized day 8 7/65×100%=11%
3. Sham, contralateral-DRG, euthanized day 8 10/46×100%=22%
4. PNI, ipsilateral-DRG, euthanized day 8 52/77×100%=66%
5. PNI, contralateral-DRG, euthanized day 8 37/51×100%=73%
6. PNI, ipsilateral-DRG, euthanized day 15 13/28×100%=49%
7. PNI, contralateral-DRG, euthanized day 15 13/44×100%=30%

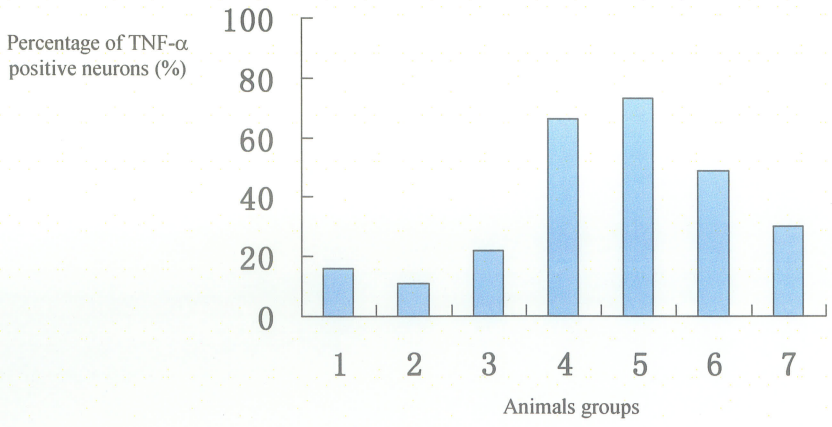
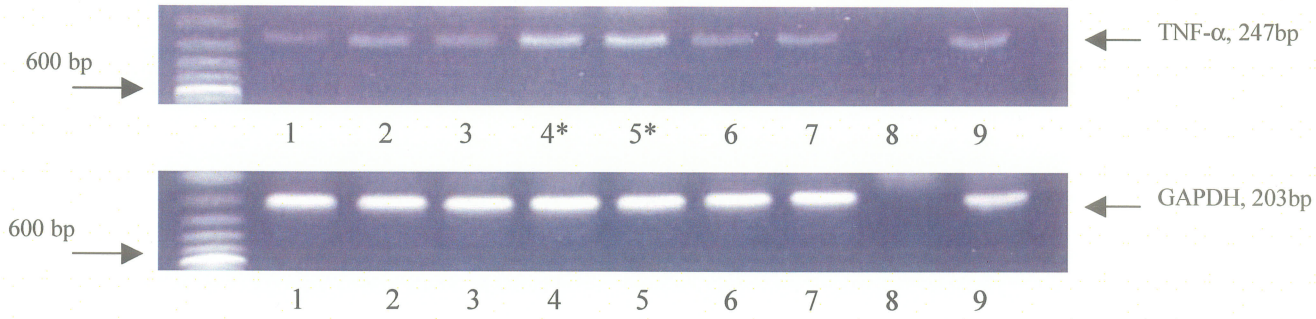
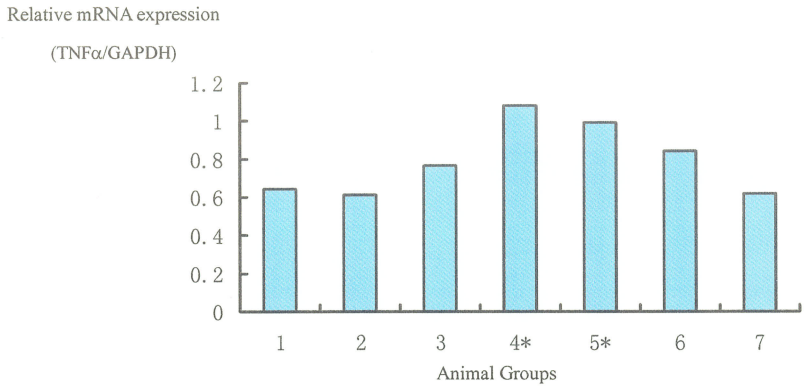


Figure 7: RT-PCR results of TNF-α expression within DRG. The signals of PNI, euthanized at day 8, both contra and ipsilateral DRG*, are significantly stronger than other groups



1. Naïve control
2. Sham operation, ipsilateral-DRG, euthanized day 8
3. Sham operation, contralateral-DRG, euthanized day 8
*4. PNI, ipsilateral-DRG, euthanized day 8
*5. PNI, contralateral-DRG, euthanized day 8
6. PNI, ipsilateral-DRG, euthanized day 15
7. PNI, contralateral-DRG, euthanized day 15
8. Water (negative control)
9. Spleen (positive control)

Figure 8: RT-PCR results of TNF-α expression within DRG. By comparing the luminosity of the TNF-α bands as a ratio to those of GAPDH shown on figure 7, relative expression of TNF-α within DRG was obtained. The expression of TNF-α of PNI, euthanized at day 8, both ipsi- and contralateral DRG*, were higher than other groups.



1. Naïve control
2. Sham operation, ipsilateral-DRG, euthanized day 8
3. Sham operation, contralateral-DRG, euthanized day 8
*4. PNI, ipsilateral-DRG, euthanized at day 8
*5. PNI, contralateral-DRG, euthanized at day 8
6. PNI, ipsilateral-DRG, euthanized at day 15
7. PNI, contralateral-DRG, euthanized at day 15

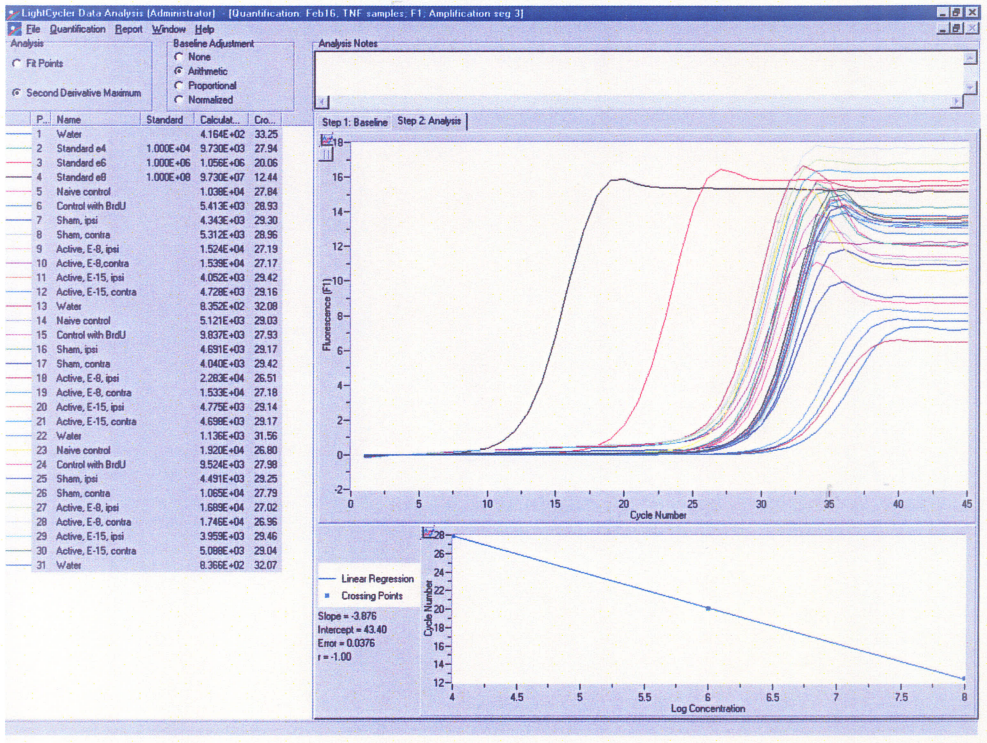


Figure 9 : Real-time PCR results of TNF-α expression. The cDNA (RT products) corresponding to each animal group was loaded to three Lightcycler capillaries, then all the samples underwent amplification with previously established standards in Real-time PCR thermo cycler. Each curve corresponded to one reading of TNF-α expression, the unit is DNA copies/ μ l.

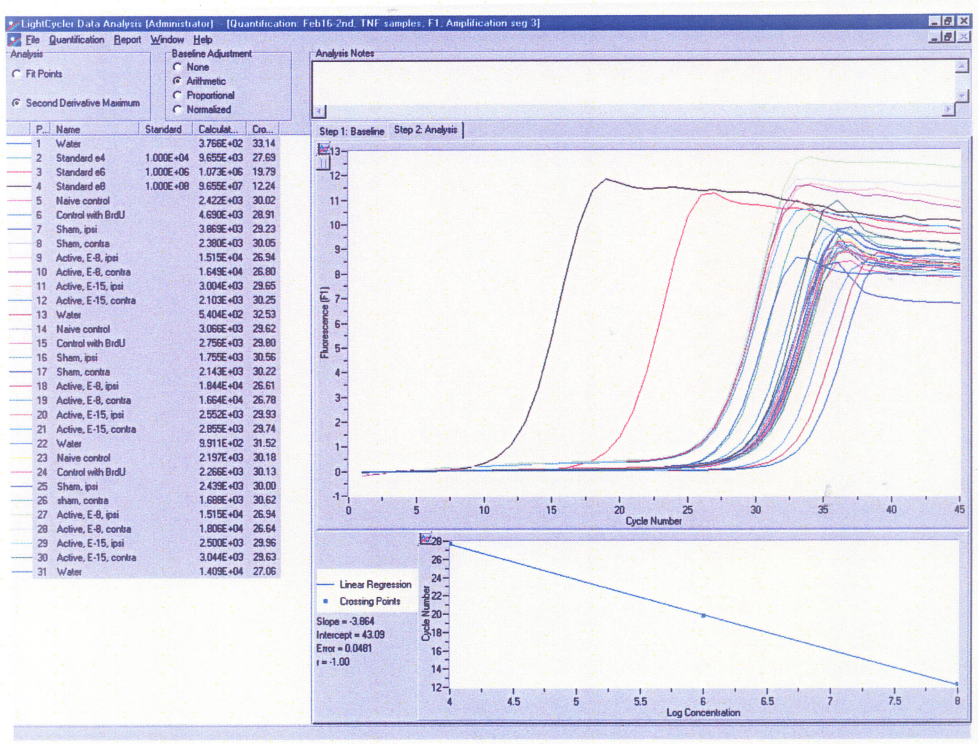
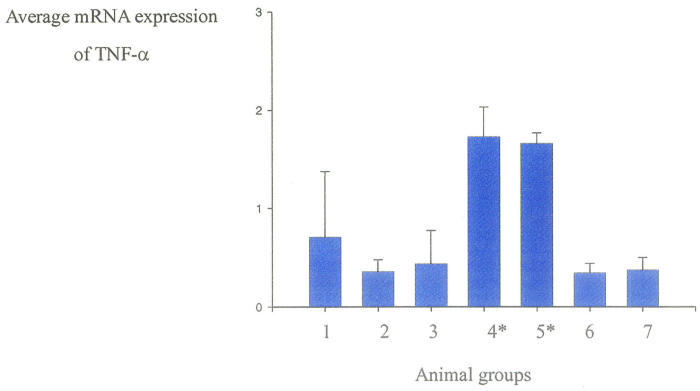


Figure 10 : Real-time PCR results of TNF-α expression. The cDNA (RT products) corresponding to each animal group was loaded to three Lightcycler capillaries, then all the samples underwent amplification with previously established standards in Real-time PCR thermo cycler. Each curve corresponded to one reading of TNF-α expression, the unit is DNA copies/ μ l.

Table 4: Real-time PCR results of TNF-α expression within DRG. Quantitative Real-time PCR data was collected from figure 9 and 10. One way ANOVA analysis was performed using statistical software SAS. The results (unit=copies/μl) indicated that the TNF-α expression of both ipsi- and contralateral DRG for animals of PNI, euthanized at day 8* was significantly higher than other groups (p<0.05, n=6). There was no statistical different between these two (p>0.05, n=6). There was no significant difference between sham and naïve control, nor between sham and PNI, euthanized at day 15 (p>0.05, n=6).

	Naïve	Sham, ipsi	Sham,contra	PNI* E-8, ipsi	PNI* E-8, contra	PNI E-15, ipsi	PNI E-15, contra
	0.2422	0.3869	0.238	1.515	1.649	0.3004	0.2103
	0.3066	0.1755	0.2143	1.844	1.664	0.2552	0.2855
	0.2197	0.2439	0.1688	1.515	1.806	0.25	0.3044
	1.038	0.4323	0.5312	1.524	1.539	0.4052	0.4728
	0.5121	0.4691	0.4040	2.283	1.533	0.4775	0.4698
	1.920	0.4491	1.065	1.689	1.746	0.3959	0.5088
Mean	0.7064	0.3595	0.4369	1.728	1.656	0.3477	0.3753
SD	0.6682	0.1211	0.3363	0.3018	0.1091	0.0925	0.1238

Figure 11: Real-time PCR results of TNF-α expression within DRG. Corresponding to the data shown in above table, the TNF-α expression of both ipsi- and contralateral DRG for animals of active lesion, euthanized at day 8* was significantly higher than other groups (p<0.05, n=6).



1. Naïve control
2. Sham operation, ipsilateral-DRG, euthanized day 8
3. Sham operation, contralateral-DRG, euthanized day 8
*4. PNI, ipsilateral-DRG, euthanized at day 8
*5. PNI, contralateral-DRG, euthanized at day 8
6. PNI, ipsilateral-DRG, euthanized at day 15
7. PNI, contralateral-DRG, euthanized at day 15

6. DISCUSSION

6.1 *Upregulation of TNF-α within DRG following axotomy*

Our results show significant TNF-α upregulation within the DRG during the initial 8 days post-axotomy. These results suggest that TNF-α is an early modulator involved in the downstream development of neuropathic pain. In this capacity, TNF-α may serve as an indirect catalyst that facilitates subsequent cellular sequences involved in the cascade of events that lead to this type of chronic pain syndrome, alternatively, TNF-α may play a direct role via its ability to induce cellular apoptosis. Irrespective of its potential pathogenic roles in neuropathic pain, any strategies targeting the attenuation of its early release during the first week post-injury may have significant clinical impact in terms of preventing the development of neuropathic pain.

6.2 *Transient upregulation of TNF-α*

Following PNI, the released TNF-α binds its receptors to exert biological functions. The receptor-bound TNF-α is rapidly internalized by monocytes and degraded intracellularly to a principal molecular species (Aranguez et al. 1995). In addition, extracellular TNF-α can be degraded by a variety of proteolytic enzymes, such as neutrophil elastase and cathepsin G (Mezyk-Kopec et al. 2005). Further, TNF-α has capacity to undergo retrograde and anterograde axonal transport following injury, which also enhances the dissipation of TNF-α from the principle site of injury (Shubayev and Myers 2002). In addition, by quantifying the radioactive signal corresponding to TNF-α mRNA, its half-life was estimated to be 44-50 minutes in macrophages culture, indicating that TNF-α is short-lived *in vitro* (Mijatovic et al.

2000). Henceforth, based on these elimination mechanisms, it is not surprising that our results depict only a transient time period of elevated TNF-α expression at day 8 post-injury. However, the significance of our research is that we were able to define a definite time period post-injury when treatment intervention is critical. As such, early treatment intervention during this narrow window of opportunity represents a critical step towards interrupting the downstream cellular events involved in the pathophysiology of neuropathic pain.

6.3 *Contralateral TNF-α upregulation*

In human, neuronal dysfunction such as mirror movements and mirror pain provide evidence for point-to-point bilateral matching of neural function (Forss et al. 2005; Merello et al. 2005). Following unilateral PNI in animal models, there are well-documented events that affect the contralateral uninjured side (Koltzenburg et al. 1999; Jaaskelainen et al. 2005). Ruohonen *et al* reported increased expression of IL-1β, transforming growth factor-β1 (TGF-β1), IL-10, and TNF-α in contralateral sciatic nerve following unilateral nerve transection (Ruohonen et al. 2002). However, other studies by Kleinschnitz *et al* demonstrated that contralateral levels of IL-1β and IL-10 were significantly increased, while TNF-α expression remained unchanged (Kleinschnitz et al. 2005). These contradictory findings may be the result of the timing of tissue analysis post-injury where the transient effects of TNF-α may not have been fully captured.

Immunohistochemical analysis and fully quantitative Real-time PCR conclusively demonstrate that TNF-α expression is upregulated in both ipsilateral and contralateral DRG

following unilateral sciatic nerve transection. However, the exact molecular and anatomical pathways that link peripheral nerve responses between both sides of the body are unclear (Kleinschnitz et al. 2005). Most likely, the damage of unilateral DRG leads to a signal transferred into the CNS, which thereby activates contralateral neurons through some sort of unknown compensatory mechanism in response to nerve injury. Plausible mechanisms for cell signaling between ipsi- and contralateral side may be explained upon advanced understanding of the complex anatomical neuronal network within the CNS and between the CNS and PNS. For example, anatomical studies suggest that the two sides of the spinal cord are connected by commissural interneurons (Matsuyama et al. 2004). These neurons remain silent or show low activity in normal conditions and become activated when the excitability of the target neurons is increased such as occurs post-injury (Sotgiu et al. 2004). After unilateral nerve injury, signals from the ipsilateral DRG could reach corresponding contralateral DRG with a precise homonymous anatomical representation by the connections of these commissural interneurons, and evoke symmetrical responses (Jankowska et al. 2005). In fact, it has been shown that the neuronal hyperexcitability following unilateral PNI can be reduced by contralateral administration of lidocaine and U50,488H, a κ-opioid receptor agonist (Bileviciute-Ljungar et al. 2001; Bileviciute-Ljungar and Spetea 2004), which may suggest a new plausible target against neuropathic pain.

6.4 *Cellular source of TNF-α expression*

Our results demonstrate that the majority DRG neurons involved in TNF-α expression following sciatic nerve axotomy are small to medium sized (15-30μm in soma diameter,

average=23.9μm, **Figure 5**), corresponding to Aδ and C nociceptive neurons. Based on our immunohistochemical analysis, there appears to be a consistent switch in TNF-α expression from predominant satellite cell and peripheral baseline neuronal source in uninjured animals to a source of predominant smaller ($p<0.05$) neurons with peripheral satellite cell background (**Figure 4, 5**). This apparent change in cellular source in response to injury correlates with our quantitative Real-time PCR data that clearly demonstrate statistically significant elevations in TNF-α expression in the injured *vs* uninjured animals. As such, our results indicate that an injury-evoked TNF-α expression from predominantly nociceptive sensory neurons represents yet another plausible target for attenuating pain.

6.5 *Limitations and future research considerations*

Besides TNF-α, other proinflammatory cytokines, such as IL-12 and IFN-γ, are found to be associated with modulation of apoptosis as well as the development of neuropathic pain (Fan et al. 2002; Song et al. 2005). IL-12 is an important regulatory cytokine that has the capacity to regulate the differentiation of naive T-cells into Th1 cells from which IFN-γ is secreted. To investigate the responses of IL-12 and IFN-γ and T-cell involvement following PNI is beneficial to better understanding of the underlying pathogenesis of neuropathic pain.

Murata *et al* reported significantly higher expression of TNF-α in the DRG at 1, 3, 7, and 14 days after experimental disc herniation surgery (Murata et al. 2004). In a tourniquet model, TNF-α upregulation was found even only one hour after injury (Mizusawa et al. 2003). However, other studies have shown that the highest expression peak of TNF-α was observed

in the contralateral endoneurium of sciatic nerve on day 35 after unilateral sciatic nerve transection (Ruohonen et al. 2002). Although our study depicts a transient upregulation of TNF-α during the first 8 days that diminished to baseline level by day 15, the variation of TNF-α expression demonstrated in other studies may be influenced by severity and type of nerve injury, or anatomical structure (DRG *vs* endoneurium of sciatic nerve) that is used to study TNF-α expression. Optimal mapping of TNF-α expression in response to injury may be better achieved by assessing animals at more frequent time intervals that cover a longer period of time. Henceforth, future research design should include more time points, such as one hour, 12 hours, 2, 3, 12, and 21 days post-injury, to better demonstrate the time-dependent changes of TNF-α expression.

In addition, in order to quantitatively and clinically evaluate the pain responses experienced by animals post-injury, pain behavior tests may be used in future studies. Pain behavior tests allow us to measure the degree of pain and pain relief by measuring animal responses and reaction time to various stimuli. One of the most popular animal models is to measure the animal's reaction time to heat (hot plate) applied to its paw, thermal nociceptive thresholds were determined using a sensitive thermal-testing device (D'Amour and Smith 1941; Abdel-Salam and El-Batran 2005; Ding et al. 2005). Another model, which is called von Frey hair testing, assesses the animal's paw withdrawal response to mechanical pressure (von Frey 1894; Duarte et al. 2005; Murata et al. 2005). Additionally, in Randall-Selitto test, the inflamed and the non-inflamed hind paw were exposed to an increasing force until the nociceptive reaction (vocalization or paw withdrawal) was reached (Randall and Selitto

1957). Elaborate electronic models of these testing devices have been developed in past decades, which have made these tests the standard methods for behavioral testing for the degree of pain (Eaton 2003).

7. CONCLUSIONS

Notwithstanding these limitations, the results of our current research indicated that following unilateral sciatic nerve transection, TNF-α expression was upregulated within both ipsi- and contralateral DRG. The upregulation of TNF-α expression occurred in a transient time period, within the first 8 days post-injury, which subsequently diminished to baseline level by day 15. In addition, the transient upregulation of TNF-α expression was associated with a switch in cellular source from a predominant baseline satellite cell source to that which was largely driven by sensory neurons.

Despite the predominant satellite cell source of TNF-α expression in naïve and sham control animals, very few large diameter neurons (average=29.0μm) also contributed to the baseline expression of TNF-α. However, when compared to the injured animals, the numerous neurons that produced TNF-α were in the smaller size (average=23.9μm, $p<0.05$). As a result, there may be a shift in the neuronal subtypes responsible for producing TNF-α in response to injury. Alternatively, the neuronal size reduction that we reported may simply be due to neuronal atrophy following peripheral nerve axotomy.

In addition, our findings also indicated that the traumatic injury to the skin and/or muscle

during sham operation did not result in the upregulation of TNF-α within DRG. This suggested that the injury of the collateral branches of peripheral nerves which innervate peripheral targets were not involved in the development of neuropathic pain, while injury to the major peripheral nerve branches, such as sciatic nerve, was proven to trigger the burst of TNF-α expression, which may subsequently result in neuropathic pain.

Based on our findings, treatment strategies of neuropathic pain should focus on timing and cellular source following injury. Specifically, in order to attenuate neuropathic pain, treatment strategies have to be designed immediately following major peripheral nerve injury within the first week. In addition, because we had demonstrated that the TNF-α upregultion were found at both ipsi- and contralateral DRG, treatment strategies should consequently focus on both sides to the injury. Futhermore, our findings revealed that there appeared to be a shift in cellular source of TNF-α expression from satellite cells to neuronal cells in response to injury. Target treatments that could deliver TNF-α inhibitors to this particular subpopulation of neuronal cells might specifically interrupt the cascade events leading to the development of neuropathic pain. Ideally, the ability to identify exact neuronal subpopulations that were responsible for injury-induced TNF-α production would be beneficial for developing specific treatment strategies against neuropathic pain.

Appendix #1

Temperature parameters setting for Real-time PCR analysis of TNF-α, using TNF-α primers shown at **Table 3**

Program 1: Pre-incubation

Cycle program Data	Value
Cycles	1
Analysis Mode	None
Temperature Targets	Segment 1
Target temperature (°C)	95
Incubation Time (min)	10:00
Temperature Transition Rate (°C/s)	20.0
Secondary Target Temperature (°C)	0
Step Size (°C)	0.0
Step Delay (cycles)	0
Acquisition Mode	None

Program 2: Amplification

Cycle program Data	Value		
Cycles	45		
Analysis Mode	Quantification		
Temperature Targets	Segment 1	Segment 2	Segment 3
Target temperature (°C)	95	59	72
Incubation Time (s)	10	5	10
Temperature Transition Rate (°C/s)	20.0	20.0	20.0
Secondary Target Temperature (°C)	0	0	0
Step Size (°C)	0.0	0.0	0.0
Step Delay (cycles)	0	0	0
Acquisition Mode	None	None	Single

Program 3: Melting curve analysis

Cycle program Data	Value		
Cycles	1		
Analysis Mode	Melting curves		
Temperature Targets	Segment 1	Segment 2	Segment 3
Target temperature (°C)	95	65	72
Incubation Time (s)	0	15	0
Temperature Transition Rate (°C/s)	20.0	20.0	0.1
Secondary Target Temperature (°C)	0	0	0
Step Size (°C)	0.0	0.0	0.0
Step Delay (cycles)	0	0	0
Acquisition Mode	None	None	Cont.

Program 4: Cooling

Cycle program Data	Value
Cycles	1
Analysis Mode	None
Temperature Targets	Segment 1
Target temperature (°C)	40
Incubation Time (s)	30
Temperature Transition Rate (°C/s)	20.0
Secondary Target Temperature (°C)	0
Step Size (°C)	0.0
Step Delay (cycles)	0
Acquisition Mode	None

Appendix #2

Raw data of TNF-α positive neurons diameter measurement

1. Naïve control

Serial number of TNF-α positive neurons	Diameter (μm)
1	39.4
2	23.2
3	31.0
4	24.6
5	33.1
6	37.3
7	17.6
8	13.4

2. Sham, ipsilateral-DRG

Serial number of TNF-α positive neurons	Diameter (μm)
1	27.9
2	37.9
3	35.0
4	21.4
5	30.7
6	16.4
7	27.1

3. Sham, contralateral-DRG

Serial number of TNF-α positive neurons	Diameter (μm)
1	37.3
2	40.1
3	23.2
4	29.6
5	31.7
6	39.4
7	23.2
8	15.5
9	33.8
10	35.2

4. PNI, ipsilateral-DRG, euthanized at day 8

Serial number of TNF-α positive neurons	Diameter (μm)	Serial number of TNF-α positive neurons	Diameter (μm)
1	27.9	27	17.9
2	17.9	28	15.7
3	24.3	29	21.4
4	10.0	30	21.4
5	13.6	31	28.6
6	14.3	32	39.3
7	23.6	33	22.9
8	19.3	34	15.0
9	17.9	35	25.7
10	24.3	36	17.1
11	17.9	37	40.7
12	11.4	38	15.7
13	27.1	39	30.0
14	26.4	40	19.3
15	25.7	41	25.0
16	20.0	42	38.6
17	16.4	43	23.6
18	40.7	44	15.0
19	25.0	45	23.6
20	32.9	46	23.6
21	23.6	47	17.9
22	35.0	48	19.3
23	14.3	49	11.4
24	13.6	50	15.0
25	20.7	51	25.0
26	15.7	52	15.7

5. PNI, contralateral-DRG, euthanized at day 8

Serial number of TNF-α positive neurons	Diameter (μm)	Serial number of TNF-α positive neurons	Diameter (μm)
1	36.6	20	22.5
2	19.7	21	39.4
3	21.1	22	31.7
4	25.4	23	31.0
5	21.1	24	13.4
6	36.6	25	35.9
7	28.9	26	26.1
8	27.5	27	24.6
9	39.4	28	16.9
10	31.0	29	13.4
11	14.8	30	33.1
12	16.2	31	21.8
13	27.5	32	26.0
14	27.5	33	23.2
15	16.2	34	38.0
16	28.9	35	43.7
17	33.8	36	9.2
18	33.1	37	19.7
19	28.2		

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