THE ROLE OF HIGH MOBILITY GROUP BOX 1 AND TOLL LIKE RECEPTOR 4

IN A RODENT MODEL OF NEUROPATHIC PAIN

Polina Feldman

Submitted to the faculty of the University Graduate School
In partial fulfillment of the requirements
for the degree
Doctor of Philosophy
in the Medical Neuroscience Graduate Program
Indiana University

February 2013

Accepted by the Faculty of Indiana University, in partial
fulfillment of the requirements for the degree of Doctor of Philosophy.

_____
Gerry S. Oxford, Ph.D., Chair

_____
Fletcher A. White, Ph.D.

Doctoral Committee

_____
Rajesh Khanna, Ph.D.

_____
Kathryn J. Jones, Ph.D.

December 11, 2012

_____
Riyi Shi, Ph.D.

DEDICATION

This thesis is dedicated to my parents, Janna and Dmitry Feldman, my brother, Alexander, and my grandparents Meri and Grigoriy Goldberg for their love, support and encouragement.

ACKNOWLEDGEMENTS

This dissertation would not be possible without the support, guidance and inspiration of a number of people. I have been extremely fortunate to have been influenced and guided by some truly remarkable people. I would like to acknowledge several people who made significant contributions to my academic training and my life.

First and foremost, I would like to thank my advisor, Dr. Fletcher A. White, for providing an encouraging environment to develop as a training scientist. I am deeply indebted to Dr. White for accepting me to his laboratory at Loyola University-Chicago and providing me the opportunity to continue here at Indiana University. I will be forever grateful to him for that. As a mentor, Dr. White has given me the freedom to explore and develop an independent research project, provided expert experimental guidance, and allowed me to further develop scientifically by providing a number of opportunities to present my findings. I thank Dr. White for challenging me to push beyond what is known and ask tough questions. Dr. White is highly respected among his colleagues and trainees and I have been very fortunate to have his guidance throughout my graduate training.

I would like to thank the members of my thesis committee Dr. Gerry Oxford, Dr. Kathryn Jones, Dr. Rajesh Khanna, Dr. Riyi Shi, for their guidance, suggestions, and constructive critiques. I would also like to thank Dr. Cynthia Hingtgen who was also a committee member, for her integral part in guiding my project and providing constructive feedback. Each of you has had a positive impact in my training and experiences that will help guide me as I progress in my

scientific career. I would like to thank Dr. Khanna for his mentorship and helping me focus my scientific questions. I would also like to thank Dr. Jones and Dr. Shi for their valuable guidance and feedback. I would especially like to thank Dr. Oxford for his insightful advice, mentorship, and for keeping me grounded and reasonable during my progression in graduate school. I am truly fortunate to have such guidance and mentorship.

My scientific growth in the last few years would not be complete without the moral support of present and past members of the White lab. I would like to especially thank Matthew Ripsch who trained me on many methods used in the lab and performed much of the tactile behavior. Without him much of the behavioral research would not be possible. I would like to thank Dr. Michael Due, who performed the electrophysiological recordings discussed in this document, for all the help during my graduate studies. I also would like to show my gratitude to past members of the White lab, Dr. Natalie Wilson, David Buchanan, and Lauren Petty.

I would like to thank the Medical Neuroscience Graduate Program and Stark Neuroscience Research Institute for providing the ability to transfer from Loyola University and continuing to support my graduate work. I would like to thank Dr. Theodore Cummins for all your guidance and encouragement. I would also like to thank the Neuroscience Graduate Program at Loyola University-Chicago for their support during my first year in graduate school. I also thank Dr. Edward J. Neafsey for all his guidance during my time at Loyola University. I

would like to thank the Graduate School of Indiana University. I especially would like to thank Dr. Patricia Gallagher for the positive outlook and support.

Additionally, I would like to thank the faculty and students in the Medical Neuroscience Program and Department of Pharmacology and Toxicology for all their feedback and critiques during Friday morning presentations and seminars. I would like to especially thank Dr. Michael Vasko and Dr. Grant Nicol for their constructive critiques and encouraging words. I would like to thank Nicole Ashpole for the kind support and positivity. I thank Joel Brittian and Sarah Wilson for their feedback in designing experiments and enjoyable personalities. I would also like to thank Karl Koelher, Dr. Andrei Molosh, Sherry Phillips, Rena Meadows, and Chao Li for making my experiences as a graduate student very memorable. I wish you all much success in your careers. I would also like to thank the staff of the Stark Neuroscience Research Institute and the Graduate School, Nastassia Belton, Brittany Veal, Miriam Barr, Alexandra Miller, and Daniel Smith for all their hard work and patience. I also thank the National Institutes of Health for the financial support during my graduate work.

I thank several individuals who influenced me to pursue a scientific career. I would like to thank Dr. Elizabeth Grove for providing me with a wonderful lab experience and support to continue to graduate school. I would also like to thank past and present members of the Grove lab, Dr. Jennifer Wilcoxon, Dr. Guliana Caronia-Brown, and Stavroula Assimacopoulos. I would like to thank those who influenced me to engage in scientific research during my undergraduate studies,

Dr. Richard Davidson, Dr. Donald MacCoon, Dr. Jolien Connor, and Dr. Albee Messing.

I am equally grateful to several friends outside the academic environment for providing encouragement and support along the way. Lastly, I would like to thank my family, my parents Janna and Dmitry Feldman, my brother Alexander and my grandparents Meri and Grigoriy Goldberg. I am very thankful for your positivity and strength especially during the writing portion. You have given me the support to purse my scientific interests and instilled the confidence in my abilities throughout this journey.

ABSTRACT

Polina Feldman

The Role of High Mobility Group Box 1 and Toll like Receptor 4 in a Rodent Model of Neuropathic Pain

Neuropathic pain is a serious health problem that greatly impairs quality of life. The International Association for the Study of Pain (IASP) defines neuropathic pain as 'pain arising as a direct consequence of a lesion or disease affecting the nervous system'. It is important to note that with neuropathy the chronic pain is not a symptom of injury, but rather the pain is itself a disease process. Novel interactions between the nervous system and elements of the immune system may be key facets to a chronic disease state. One of particular note is the recent finding supporting an interaction between an immune response protein high mobility group box 1 (HMGB1) and Toll like receptor 4 (TLR4). HMGB1 is an endogenous ligand for TLR4 that influences the induction of cytokines in many non-neuronal cells. After tissue damage or injury, HMGB1 may function as a neuromodulatory cytokine and influence the production of pro-nociceptive mediators altering the state of sensory neurons. Very little is known about the HMGB1-TLR4 interaction in sensory neurons and whether chronic changes in endogenous HMGB1 signaling influence the establishment of neuropathic pain. This thesis aims to determine whether a physiologically relevant neuroimmune interaction involving endogenous HMGB1 and TLR4 in the dorsal root ganglia is altered following a tibial nerve injury model of neuropathic pain. I hypothesized that sensitization of sensory neurons following a peripheral nerve injury is dependent on endogenous HMGB1 and TLR4.

The studies presented here demonstrate that HMGB1 undergoes subcellular redistribution from the nucleus to the cytoplasm in primary afferent neurons following peripheral nerve injury. Further, the presence of extracellular HMGB1 may directly contribute to peripheral sensitization and injury-induced tactile hyperalgesia. Though thought to be important as a pivotal receptor for HMGB1 activation, neuronal protein expression of TLR4 does not appear to influence the effects of HMGB1-dependent behavioral changes following peripheral nerve injury. Taken together, these findings suggest that extracellular HMGB1 may serve as an important endogenous cytokine that contributes to ongoing pain hypersensitivity in a rodent model of neuropathic pain.

Gerry S. Oxford, Ph.D., Chair

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVATIONS

ALS	Amyotrophic lateral sclerosis
AP	Action potential
ATF3	Activating transcription factor 3
ATP	Adenosine 5'-triphosphate
BL	Baseline
BSS	Balanced sterile solution
C	Cysteine
CaMK	Calcium/calmodulin-dependent protein kinase
CCI	Chronic constriction injury
CCL	Chemokine (C-C motif) ligand
CCR2	C-C chemokine receptor type 2
CFA	Complete Freund's adjuvant
CNS	Central nervous system
CGRP	Calcitonin gene related peptide
CXCL	Chemokine (C-X-C motif) ligand
CXCR	Chemokine (C-X-C motif) receptor
DAMP	Damage associated molecular pattern
DRG	Dorsal root ganglion
ER	Endoplasmic reticulum
GA	Glycyrrhetinic acid
GABA	Gamma-aminobutyric acid

GL	Glycyrrhizin
GDNF	Glial cell line-derived neurotrophic factor
HMGB1	High mobility group box 1
HSP	Heat shock protein
IASAP	International Association for the Study of Pain
IB4	Isolectin B4
ICV	Intracerebroventricular
IFN	Interferon
IL	Interleukin
IRG1	Immunoresponsive gene 1
IP	Intraperitoneal
IPL	Intraplantar
IR	Immunoreactivity
IRAK	IL-1 receptor associated kinase
IRF	Interferon regulatory factor
IT	Intrathecal
IV	Intravenous
JNK	c-Jun N-terminal kinases
LPS	Lipopolysaccharide
LTP	Long-term potentiation
M3G	Morphine-3-glucuronide
MAPK	Mitogen-activated protein kinase

MCP1	Monocyte Chemotactic Protein 1
MD2	Myeloid differentiation protein-2
MyD88	Myeloid differentiation primary response protein 88
NaV	Voltage-gated sodium channel
NeuPSIG	Special Interest Group on Neuropathic Pain
NGF	Nerve growth factor
NFkB	Nuclear factor κB
NLS	Nuclear localization sequence
NMDAR	N-methyl-D-aspartate receptor
PAMP	Pathogen associated molecular pattern
PID	Post injury day
PNS	Peripheral nervous system
PKC	Protein kinase C
PWT	Paw withdrawal threshold
RAGE	Receptor for advanced glycation end products
ROS	Reaction oxygen species
SARM1	(SAM) and an Armadillo repeat motif (ARM) 1
SC	Subcutaneous
SD	Sprague Dawley
SDF	Stromal derived factor
SGC	Satellite glial cells
SIGIRR	Single-Ig-interleukin 1 related receptor

SNL	Spinal nerve ligation
SNI	Spared nerve injury
TAG	TRAM adaptor with GOLD domain
TIR	Toll/interleukin 1 receptor
TIRAP	TIR domain-containing adapter protein
TLR	Toll like receptor
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TNI	Tibial nerve injury
TOLLIP	Toll-interacting protein
TRG	Trigeminal ganglia
TRAF	TNF receptor-associated factor
TRAM	TRIF-related adaptor molecule
TRIF	TIR domain-containing adapter inducing interferon-β
TRP	Transient receptor potential
TRPV1	Transient receptor potential channel subfamily V member 1
VGCC	Voltage-gated calcium channel
VGCs	Voltage-gated channels
VGSC	Voltage-gated sodium channel

CHAPTER I

INTRODUCTION

A. Pain

The International Association for the Study of Pain (IASAP), defines pain as ‘an unpleasant sensory and emotional experience associated with actual or potential tissue damage’ (Merskey, 1994). As a sensory experience, pain is often categorized as “good pain” -nociceptive pain, or “bad pain” -pathophysiological pain (Devor, 2006). As an emotional (affective) experience, pain is a moment-to-moment conscious and cognitive process that brings forth feelings of fear and suffering and often times results in withdrawal and avoidance behaviors (Price, 2000).

In the most primitive form, pain functions as an early warning mechanism to detect and protect against potentially tissue-damaging noxious stimuli. Because this pain has a direct behavioral response to sensing noxious stimuli (i.e. rapid withdrawal reflex), it is often referred to as nociceptive pain (Woolf, 2010). Pain can also function as a critical indication for proper recovery after injury or illness. Acute pain is a state that is adaptive, protective and encourages the healing process. For instance, inflammatory pain is an acute pain state that is caused by the activation of the immune system during tissue injury or infection and serves as a protective response (Devor, 2006). The etiology of acute pain

may arise from a number of medical conditions; however, when pain becomes chronic condition, it can be considered a disease (Costigan *et al.*, 2009).

A chronic pain state is no longer protective, but instead maladaptive, resulting from abnormal neurobiological processing in the nervous system. Chronic pain can arise from damage to the nervous system (neuropathic pain) but can also arise without damage to the nervous system or inflammation (dysfunctional pain) (Costigan *et al.*, 2009). Each pain state, whether acute or chronic is highly subjective, depends greatly on past pain experience, physical health, psychological/mental status, genetic predisposition, and current pain phenotype (von Hehn *et al.*, 2012).

1. Types of pain

Pain can be viewed on a spectrum ranging from acute to chronic. At first, acute pain is experienced rapidly in response to disease or injury and serves to alert the body that something is wrong and responds accordingly, such as removing your hand from a hot stove. Acute pain often resolves within a short time once the underlying condition is treated. However, acute pain can be a sign of a serious disease or condition. A good example is cardiac pain or angina pectoris that precipitates moments leading up to a myocardial infarction. When nerves from the damaged heart tissue convey pain signals to spinal cord levels at thoracic (T) T1-T4 on the left side, they happen to converge with nerve fibers from the throat, neck and left arm at the same spinal cord levels and pain is transmitted and felt in those somatic structures (Foreman, 1999). Crushing,

burning, or squeezing pain sensations are felt that can radiate to the throat, neck, and left arm. Pain that originates in one part of the body but felt in another part of the body is known as referred pain.

Unlike somatic pain that arises from muscles, bones and other soft tissues, visceral pain arising from problems with internal organs, such as the heart, liver, gall bladder, kidney, or lungs, is generally referred, diffuse, and poorly localized to the source (Sikandar & Dickenson, 2012). Moreover, pain may also act as a signal of disease, such as cancer. For instance, pain is present in 30-50% of all cancer patients (Mercadante & Arcuri, 1998) and the frequency and intensity of pain tends to increase with advancing stages of cancer (Portenoy *et al.*, 1999).

Progressing along the spectrum of pain, chronic pain often begins as acute pain continues to linger beyond the natural course of healing or after steps have been taken to address the cause of pain. Chronic pain is defined as lasting more than three months beyond the initiation of pain (Turk & Okifuji, 2009) and may develop from localized tissue damage, acute injury/disease (e.g. osteoarthritis) or damage to the nervous system-neuropathic pain (e.g. painful diabetic neuropathy, post-stroke pain, spinal cord injury) (Turk *et al.*, 2011).

2. Chronic pain symptoms and burden

Clinically, chronic pain symptoms are described as spontaneous, shooting, burning, or aching pain. For instance, neuropathic pain is a subset of chronic pain caused by damage to the nervous system and is often perceived as tingling, burning, and pins-and-needles sensations called paresthesias (Baron, 2009).

Patients report some degree of discomfort, soreness, tightness and stiffness. Pain can also lead to other symptoms such as fatigue, sleeplessness, irritability, and a weakened immune system (Turk *et al.*, 2011). Additionally, chronic pain is highly associated with other chronic co-morbidities including depression and anxiety (Berger *et al.*, 2004). The daily quality of life is greatly compromised and individuals can become impatient, hopeless, and unmotivated. Often times the symptoms are so debilitating that daily activities, such as going to work/school, can no longer carry on. In 2008, the Medical Expenditure Panel Survey estimated about 100 million adults in the United States were affected by some degree of chronic pain with the estimated national total health care costs of $560-$635 billion annually in the United States (Gaskin & Richard, 2012). Given the magnitude of the economic costs of pain, understanding the mechanisms of chronic pain to develop effective pain management therapies are needed to reduce the impact of pain on society.

3. Pain treatment

Pharmaceutical drugs are by far the most popular treatment for chronic pain. In 2010, the annual cost of pharmaceuticals for pain management was $16.4 billion (Gaskin & Richard, 2012). Antidepressant drugs, such as tricyclic antidepressants (TCAs)- amitriptyline and cyclobenzaprine, selective serotonin reuptake inhibitors (SSRIs), and selective serotonin and noradrenaline reuptake inhibitors (SNRIs), are widely used for the treatment of neuropathic pain, lower pack pain, and fibromyalgia. In some clinical studies, beneficial effects have been

reported; however, side-effects and toxicity reduce their functional effect (Verdu *et al.*, 2008). Although nonsteroidal anti-inflammatory drugs (NSAIDs) are widely prescribed for most pain states, NSAIDs are less effective for neuropathic pain and fibromyalgia (Vo *et al.*, 2009). Opioids are generally the gold standard for pain management; however, given their adverse side effects are now reserved for a second- or third- line treatment for chronic pain (Dworkin *et al.*, 2010). Anticonvulsant drugs- gabapentin, pregabalin, and carbamazepine or oxcarbazepine- has had some success for the treatment of chronic pain and display greater efficacy in neuropathic pain patients (Dworkin *et al.*, 2010; Finnerup *et al.*, 2010).

Other treatments for chronic pain may involve skeletal muscle relaxants, topical agents, nerve blocks, implantable devices, and alternative/holistic medicine. Of all the treatments available for chronic pain, the best evidence for pain reduction averages roughly 30% in about half of treated patients (Turk *et al.*, 2011). Thus a great need exists for personalized medicine to determine the effectiveness of combining pain treatments and generating novel therapeutic targets that account for the variability in chronic pain phenotypes.

B. Sensory neuron

The sensation of pain begins with the primary afferent neuron. The cell body of primary afferent neurons is located lateral to the spinal cord in the dorsal root ganglion (DRG). The primary afferent neuron (or sensory neuron) conveys thermal, mechanical, or chemical information from the physical environment to

the dorsal horn of the spinal cord. Primary afferent fibers can be distinguished by fibre conduction velocity that further correlate with fiber cross-sectional diameter (Harper & Lawson, 1985b). Primary afferent neurons are categorized as either A- (myelinated) and C- (unmyelinated) fibres. A- (myelinated) fibers comprise large Aα- (12-20μm), medium Aβ- (6-12μm), and small Aδ- (4-36μm) fibers that have a rapid conduction velocity of 72-120m/s, 36-72m/s, and 4-36m/s, respectively. C- (unmyelinated) fibers have the slowest conduction velocity at 0.4-2m/s with the smallest cross-sectional diameter (0.2-1.5μm) (Bessou & Perl, 1966; Boyd & Kalu, 1979). Primary afferent fibers also correlate with the size of the primary afferent cell body; Aβ-fibers correspond to large cell body diameters (>40μm), Aδ/C fibers correspond to medium (30-40μm), and small (<30μm) sized cell bodies (Harper & Lawson, 1985a).

Primary afferent neurons transduce mechanical, thermal, proprioceptive, and chemical information by specialized receptors. Those that innervate the skin may contain peripheral nerve endings that are encapsulated by a non-neural structure to mediate somatic modalities of touch, stretch, and proprioception (e.g. Meissner's corpuscle or Pacinian corpuscle) (Munger & Ide, 1988). Aβ-fibers terminate in the dermis or epidermal layer of the skin depending on the peripheral nerve ending (Lumpkin & Caterina, 2007). Mainly, innocuous sensory information is transmitted by low threshold (high sensitivity) mechanoreceptors that are innervated by Aβ- and some Aδ-fibers. Additionally, innocuous stimuli light, such as pleasant touch may be represented by low threshold C-fibers mechanoreceptors (Olausson *et al.*, 2002; Fang *et al.*, 2005). Whereas, pain and

thermal information are transmitted by high threshold (low sensitivity) mechanoreceptors, nociceptors, and thermoreceptors innervated by Aδ/C fibers (Kruger *et al.*, 1981; Light & Perl, 2003). Aδ-fibers innervate the dermis and parallel to the epithelial surface, whereas C-fibers innervate different epidermal layers of glabrous and hairy skin (MacIver & Tanelian, 1993b; a).

1. Nociceptive sensory neuron

There are several classes of nociceptors: mechanical, thermal, mechano-thermal, polymodal, and silent. Mechanical nociceptors are high threshold mechanoreceptors that correspond to Aδ-fibers and respond to extreme mechanical perturbations to the skin felt as sharp, pricking sensation (Cain *et al.*, 2001). Thermal nociceptors are activated by extreme temperatures (> 45°C or < 5°C), which most commonly correspond to Aδ/C fiber conduction velocity (Burgess & Perl, 1967; Lynn & Carpenter, 1982).

Mechano-thermal Aδ nociceptors are further functionally subdivided into two categories, Type I and Type II. Type I Aδ-nociceptors can be activated by intense mechanical stimuli or noxious heat at temperatures greater than 52°C (Meyer & Campbell, 1981; Treede *et al.*, 1995). Type II Aδ-nociceptors are also activated by mechanical and heat stimuli but at a lower temperature threshold of 43°C, similar to that of nociceptive C-fibers (Beitel *et al.*, 1977; Meyer & Campbell, 1981; Leem *et al.*, 1993; Treede *et al.*, 1995). Moreover, there is evidence to suggest that a fraction of A-fiber nociceptors may also conduct in the Aβ conduction velocity range (Lawson, 2002; Djouhri & Lawson, 2004).

Polymodal nociceptors respond to high intensity mechanical, chemical, or thermal stimuli and mostly correspond to C-fiber conduction velocity (~72% of polymodal nociceptors) (Lynn & Carpenter, 1982; Lawson *et al.*, 2008). For instance, C-polymodal fibers responsive to noxious heat (~10% of C-nociceptors) are presented as a slow, burning pain (Croze *et al.*, 1976; Schmidt *et al.*, 1995; Dubin & Patapoutian, 2010). Finally, silent nociceptors are activated by chronic stimulation (tissue damage, inflammation), and unresponsive during brief noxious stimuli (Neugebauer *et al.*, 1989; Handwerker *et al.*, 1991; Schmidt *et al.*, 1995).

2. Sensory transduction

The seminal work of Max Von Frey revealed that discrete areas of the skin yielded different sensory experiences (pressure, cold, warmth, and pain) and corresponded to structurally defined neural endings (Perl, 2007). Recent investigations have begun to uncover the cellular and molecular events that generate somatosensation. A range of ion channels present on peripheral nerve endings have been identified that mediate the transduction of sensory signals into electrical signals, which include transient receptor potential (TRP) channels, two pore potassium channels-TREK, P_2X_3 ATP-gated cation channels, and other voltage-gated channels (VGCs) (Patel *et al.*, 1998; Bertrand & Bornstein, 2002; Chung & Caterina, 2007; Morris & Juranka, 2007).

Most notably, the large family of TRP channels is tuned to a particular temperature range. For instance, acute thermal nociception in healthy skin involves the transient receptor potential cation channel subfamily V member 1

channel (TRPV1). TRPV1 was identified as the receptor for capsaicin (main active component of spicy peppers) to which activation of channel may occur at temperatures greater the 42°C. (Caterina *et al.*, 1997; Caterina & Julius, 2001). On the other hand TRPM8, ion channel specific to C-fibres, is activated by cool temperatures (about 30-32°C) and menthol (McKemy *et al.*, 2002; Peier *et al.*, 2002).

3. Cytochemical classification

Primary afferent neurons are either A- (myelinated) or C- (unmyelinated) fibres, were initially distinguished by Nissl staining. Dorsal root ganglion sensory neurons with large 'light' myelinated neurons display uneven staining with lightly stained cytoplasmic regions due to the abundance of interspersed neurofilaments, whereas 'small dark' unmyelinated neurons had dense staining (Lawson *et al.*, 1974; Sommer *et al.*, 1985). Antibodies against neurofilament 200 and peripherin have had some specificity to 'light' and 'small dark' sensory neurons in the rat, respectively (Lawson *et al.*, 1984; Ferri *et al.*, 1990). However, neurofilament antibodies display a varying degree of colocalization making it difficult to classing sensory neurons (Goldstein *et al.*, 1991).

Several cell surface, cytoplasmic, and enzymatic markers that delineate sensory neuron subpopulations of small nociceptive sensory and large non-nociceptive neurons have been identified (Jessell & Dodd, 1985; Snider & McMahon, 1998). Antibodies against TRPV1 have specificity to small and medium neurons (Aδ/C fibers) and do not display immunoreactivity in large

primary afferent neurons (Aβ-fibers) (Caterina *et al.*, 1997). Antibody LA4, directed against α-galactose and α-fucose extended glycolipid also labels small DRG neurons (Dodd & Jessell, 1985; Alvarez *et al.*, 1991).

Most C-fibers fall into one of two categories, one population that contains pro-inflammatory peptides-peptidergic and the other population are non-peptidergic (Snider & MacMahon, 1998). The pro-inflammatory peptidergic population is identified by peptides, calcitonin gene-related (CGRP) peptide (40% of DRG neurons CGRP-positive) and substance P (20% of DRG neurons) (Hokfelt *et al.*, 1976; Ju *et al.*, 1987). Somatostatin also labels a similar subpopulation as substance P (Hokfelt *et al.*, 1976). Peptidergic DRG neurons are dependent on nerve growth factor (NGF) for neurotrophic support during embryogenesis and postnatal survival (Molliver *et al.*, 1997).

The nonpeptidergic population can also be identified histologically by the presence of specific enzyme fluoride-resistant acid phosphatase or binding sites for isolectin B4 (IB4) (30% of DRG population IB4-positive) (Knyihar-Csillik & Csillik, 1981; Silverman & Kruger, 1990). However, experimental finding using fluoride-resistant acid phosphatase displays differences in relative numbers and distribution (Hunt & Rossi, 1985). On the other hand, IB4 is derived from the plant *Griffonia simplicifolia* that preferentially binds to nonpeptidergic small DRG neurons through α-d-galactose carbohydrate residues on their soma membranes (Silverman & Kruger, 1990; Fullmer *et al.*, 2004), however only one study has identified IB4 in both Aδ- and C-fibers (Gerke & Plenderleith, 2001). Although IB4 subpopulation may not necessarily represent a specific functional subpopulation

of sensory neurons, IB4 subpopulation co-localizes with functional ATP-activated purinergic P_2X_3 receptor (67.5% IB4 and P_2X_3 colocalization) providing an additional marker for nonpeptidergic population (Bradbury *et al.*, 1998) and high voltage-gated sodium channel (NaV)1.9 expression (Fang *et al.*, 2006). Recent studies have identified Mas-related G-protein coupled receptor member D (MRGPRD), a G-protein coupled receptor, to define a population of non-peptidergic nociceptive C-fibres in the mouse (Zylka *et al.*, 2005). While nonpeptidergic neurons are NGF-dependent during embryogenesis, their neurotrophic dependence switches during early postnatal life and instead require glial cell line-derived neurotrophic factor (GDNF) (Molliver *et al.*, 1997; Bennett *et al.*, 1998).

The differences in neurotrophic dependency is further identified by the major discovery of TrkA- the high affinity receptor for NGF, which is expressed in mainly peptidergic CGRP and/or substance P populations independent of somatostatin, IB4, or LA4 binding afferent (Verge *et al.*, 1989; Averill *et al.*, 1995; Molliver *et al.*, 1995; Kashiba *et al.*, 1996). Whereas, GDNF -dependent subpopulations express a GDNF receptor Ret- (rearranged in transformation- a tyrosine kinase transmembrane receptor) and maintain the IB4 binding phenotype and somatostatin expression in adult DRG neurons (Bennett *et al.*, 1998; Bennett *et al.*, 2000).

Furthermore, activated or injured primary afferent neurons are often identified by an additional set of neurochemical markers. Activating transcription factor 3 (ATF3), neuron specific enolase, and heat shock protein (HSP) 72 are a

neuronal markers of nerve injury or damage (Gonzalez *et al.*, 1989; Hans *et al.*, 1993; Tsujino *et al.*, 2000). c-FOS, cytochrome oxidase is a marker for metabolic neuronal activity or stimulation (Wong-Riley & Kageyama, 1986; Bullitt, 1990). Fluoro-Jade B is a high affinity fluorescent marker of neurodegeneration (Schmued & Hopkins, 2000). Taken together, these markers provide useful tools to distinguish subpopulations of DRG neurons, however many variables, such as, species, age, treatment condition may affect their specificity (Alvarez & Fyffe, 2000).

C. Nociceptive pathway

Pain is transmitted from the periphery by nociceptive primary afferent neurons to the dorsal horn of the spinal cord. The spinal cord is the first relay site in the transmission of nociceptive information from the periphery to the brain. The signal is further transmitted to higher centers in the brain, where noxious signals can be perceived as pain **(Figure 1)**.

The cell body of primary afferent neurons is located lateral to the spinal cord in the DRG. Given this unique position, primary afferent neurons have morphologically evolved as pseudounipolar neurons with a single axon that projects bi-directionally, with one branch projecting the dorsal horn of the spinal cord and another branch projecting to peripheral tissues, such as skin, muscle, and viscera. Nociceptors on sensory neuron terminals transduce painful thermal, mechanical, chemical stimuli into electrical energy to transduce the signal to neurons and interneurons residing in the dorsal horn of the spinal cord (Woolf,

2010). The speed at which the signal is transmitted is related to the diameter of the fiber. The small-diameter and slow-conducting fibers, C-unmyelinated fibers and Aδ-myelinated fibers generally transmit signals from nociceptors (Bessou & Perl, 1966). Primary afferent neurons terminate in dorsal horn of the spinal cord and release the excitatory neurotransmitter glutamate to activate second order neurons and interneurons in the dorsal horn of the spinal cord.

Primary afferent neurons project to the ipsilateral dorsal horn of the spinal cord to defined regions. Anatomical laminar organization of the dorsal horn is divided into a series of Laminae (I-V) that receive input from primary afferent neurons; Lamina (L)I- *marginal zone*, LII- *substania gelatinosa*, LIII-IV- *nucleus proprius*, and LV (Rexed, 1952; Brown, 1983). Lamina II is further subdivided into LIIo and LIIi. Nociceptive and thermoreceptive Aδ/C fibers innervate LI-II and Aδ/β myelinated low-threshold fibers innervate much of LIIi-V (Light & Perl, 1979; Brown *et al.*, 1981; Shortland *et al.*, 1989; Lawson *et al.*, 1997). Specifically, Aδ-nociceptors innervate LI with some branches extending to V, X (Light & Perl, 1979). Aδ/C peptidergic fibres innervate LI-IIo (Lawson *et al.*, 1997), where as C-non-peptidergic fibres innervate mainly LIIi (Zylka *et al.*, 2005; Lorenzo *et al.*, 2008). Overall, nociceptive primary afferent neurons convey ‘nociceptive’ information to second order neurons and polysynaptic interneurons in the spinal cord, which are essential for processing noxious information as a conscious experience and eliciting a nociceptive withdrawal reflex.

Second order neurons cross the midline in the spinal cord and project via the spinothalamic tract to the thalamus and cortical areas (somatosensory cortex,

cingulate cortex, and insula) generating the perception of pain (Price, 2000). At the same time, nociceptive information activates interneurons (inhibitory or excitatory) in the spinal cord to facilitate a nociceptive motor withdrawal reflex, via motor neurons (Todd, 2010), resulting in avoidance and protective behavior. Descending control from midbrain periaqueductal gray (PAG), rostroventral medulla, and locus coeruleus modulate spinal dorsal horn neurons (Millan, 2002). Overall, the nociceptive information conveyed at the level of the spinal cord is dependent on the activity of primary afferent neurons, the influence of local excitatory and inhibitory interneurons, and descending input from the brainstem (D'Mello & Dickenson, 2008).

Figure 1:

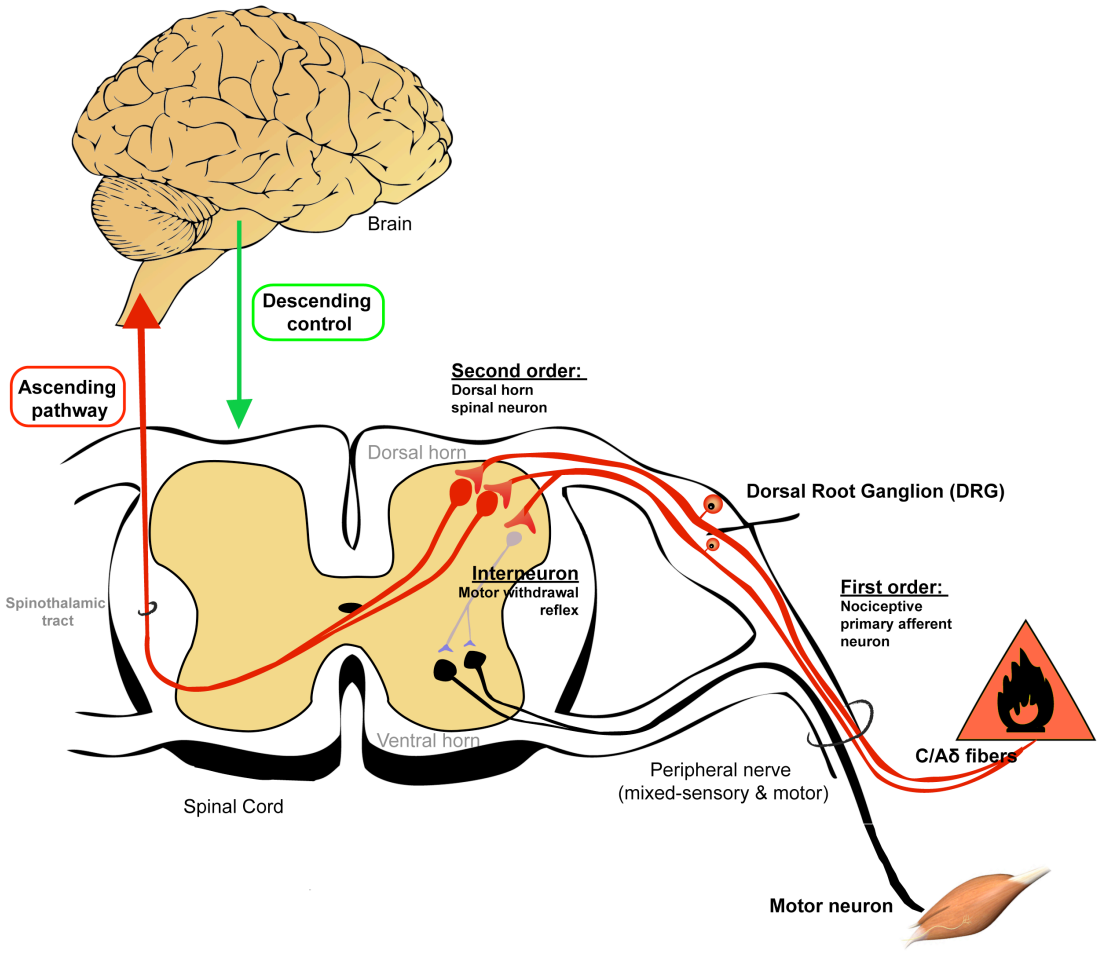


Figure 1: Nociceptive Pathway. Noxious stimuli evoke the pain pathway stimulating nociceptive primary afferent neurons. Nociceptive neurons comprise Aδ/C fibers that transduce the nociceptive signal into an electrical signal. The cell body of a primary afferent neuron is located in the dorsal root ganglion (DRG). First order, primary afferent neurons terminate onto second order neurons and interneurons in Laminae I-II of the dorsal spinal cord. In the presence of noxious stimuli polysynaptic spinal interneurons elicit motor reflexes via motor neurons of the ventral horn of the spinal cord. Second order neurons transmit the pain signal along the spinothalamic tract and synapse in the ventrolateral nucleus of the thalamus that further project to cortical areas; somatosensory cortex, cingulate cortex, and insula. Descending control originates from the midbrain; peri-aqueductal gray (PAG), rostroventral medulla, and locus coeruleus to inhibit spinal dorsal horn neurons. Spinal cord *adapted from SpringerImage*, Muscle image-*3Dscience.com,* brain clip art-*clker.com,* Fire-Hazard-Warning-Sign *adapted from Featurepics.com-490091.jpg*

D. Neuropathic pain

The Institute of Medicine reports approximately 21 million American adults suffer from neuropathic pain (Toth *et al.*, 2009). Neuropathic pain as defined by the IASP Special Interest Group on Neuropathic Pain (NeuPSIG) as 'pain arising as a direct consequence of a lesion or disease affecting the somatosensory system' (Treede *et al.*, 2008). The etiology of neuropathic pain is quite diverse ranging from metabolic disorders, direct injuries, infections, and exposure to neurotoxins (von Hehn *et al.*, 2012). Often times diagnosing neuropathic pain is quite challenging, recently a clinical grading system has been devised to aid in determining with certainty the presence or absence of neuropathic pain **(Figure 2)**. The grading system takes into account neuroanatomical pain distribution, history of lesion or disease, pain symptoms, and diagnostic tests to diagnosis neuropathic pain (Geber *et al.*, 2009).

Figure 2:

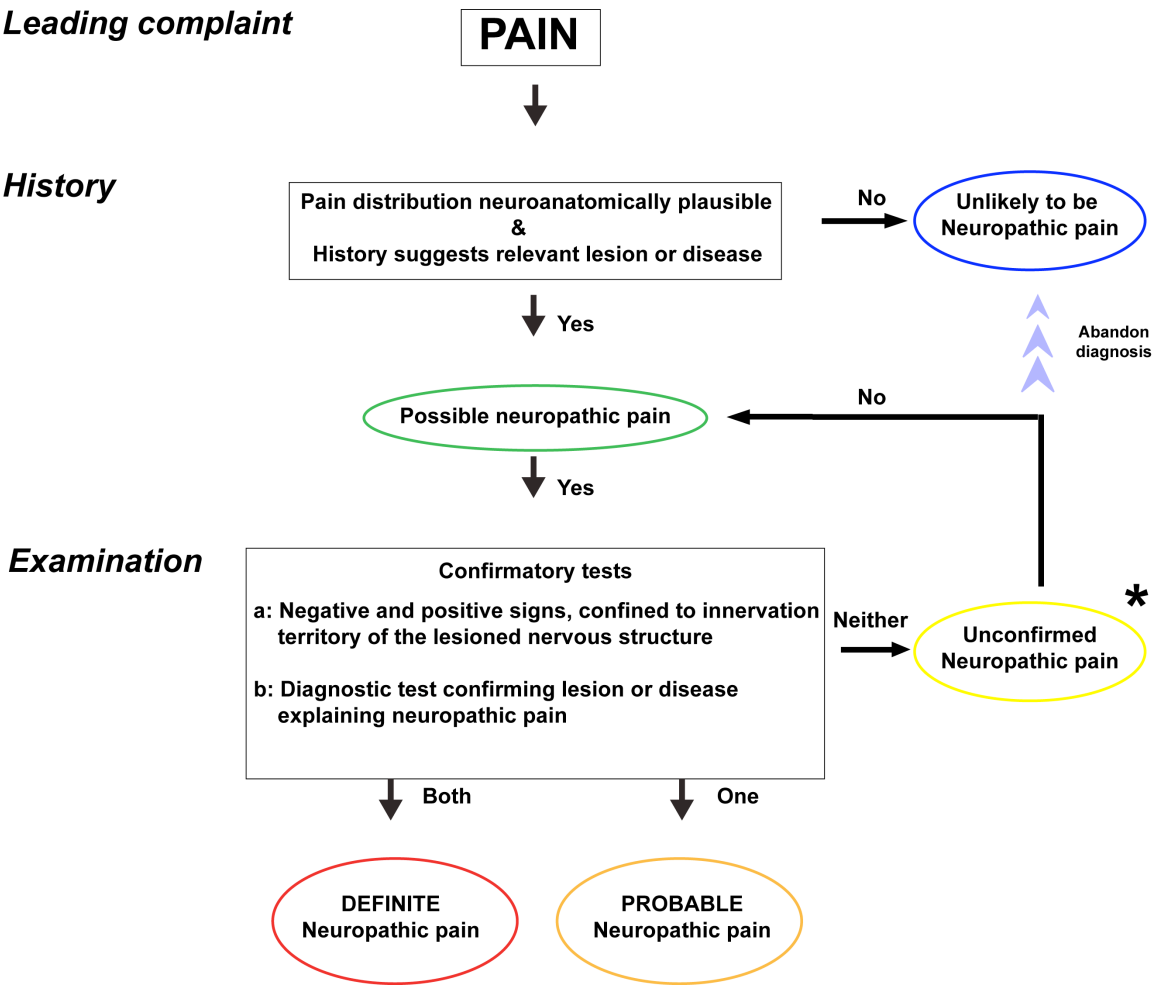


Figure 2: Flow chart of clinical grading system for neuropathic pain. The grading system can be used to decide with a level of certainty the presence or absence of neuropathic pain in an individual patient. *Additional testing continues either immediately or in later follow-up visits is necessary to obtain a diagnosis. Adapted from (Treede *et al.*, 2008; Geber *et al.*, 2009).

1. Pain symptoms, sensitivity, and nociception

Negative symptoms or deficit in function (i.e. numbness after nerve injury) are the first indication of damage to nervous system. Negative symptoms reflect reduced impulse conduction in the neural tissues, and include hypoesthesia -a decreased sensitivity and weakness. Neuropathic pain patients often report positive symptoms, such as, deep and aching sensation (i.e. nerve trunk pain). Dysesthetic pain is usually presented and described as an unfamiliar or abnormal sensation such as burning, tingling, electric, searing, drawing, or crawling. Positive symptoms reflect an abnormal level of excitability in the nervous system and include pain, paresthesia, dysesthesia, and spasms (Baron, 2009). Spontaneous pain is non-evoked and is one of the most prevalent clinical symptoms in neuropathic pain (Backonja & Stacey, 2004). For instance, patients with trigeminal neuralgia experience minimal sensory loss but experience a vast degree of spontaneous pain. Collectively, negative symptoms, such as sensory loss may be mild and generally overshadowed by positive symptoms, such as allodynia, hyperalgesia and hyperpathia, and paroxysmal spontaneous pain.

Pain sensitivity can be described as allodynia or hyperalgesia. First, allodynia is a type of hypersensitivity that is defined by the IASP as ‘pain due to a stimulus that does not normally provoke pain’ (Merskey, 1994) or pain in response to non-nociceptive stimuli. Second, hyperalgesia is defined by IASP as increased pain from a stimulus that normally provokes pain (Merskey, 1994) or an increased pain response to a nociceptive stimulus. Hyperalgesia and allodynia are key features of nociceptive pain are further classified according to

the type of stimulus; thermal (heat or cold) stimuli or mechanical brush, pinch, or pressure stimuli are most often used. Weakly myelinated (Aδ-fibers), unmyelinated (C-fibers) high-threshold fibers and few heavily myelinated Aβ-low threshold fibers convey nociceptive pain information (Djouhri & Lawson, 2004). According to the IASP task force, all forms of pain amplification including lowering in thresholds to stimuli are considered hyperalgesia. When low-threshold fibers are involved the term allodynia can be used, however, the term hyperalgesia may also be applied if it is not known whether low- or high-threshold sensory nerve fibers are involved (Sandkuhler, 2009). **Table 1** provides definitions of pain terminology.

However, pain is a conscious experience that is a highly complex, subjective experience accompanied with obligatory affective, autonomic, and cognitive components (Barrot, 2012). In patients, pain is most often assessed and quantified by verbal expression, which is not possible in rodents. Nociceptive pain assessments that measure hyperalgesia and allodynia provide an objective analysis of the nociception. Nociception includes the mechanisms by which noxious stimuli are detected by the peripheral nervous system, encoded, transferred, and unconsciously interpreted by the nervous system (Barrot, 2012). In response to peripheral damage to the nervous system, pain threshold decreases, pain hypersensitivity persists, and duration and amplitude to noxious stimuli are amplified in nociceptive neurons (Costigan *et al.*, 2009). Thus, when studying neuropathic pain assessing the mechanisms that are altered in

nociception may become a critical component for the overall conscious perception of pain.

Many nociceptive tests are utilized in accessing animal models of neuropathic pain. Evoked nociceptive pain can be measure using thermal and mechanical stimuli. Thermal stimulation includes; a tail flick test, the hot- or cold-plate tests, and the radiant heat paw-withdrawal test (Le Bars *et al.*, 2001). Nociceptive tests can also rely on the stimulus threshold necessary to elicit an avoidance or withdrawal behavior. These tests utilize mechanical stimulation and include the von Frey filaments, the Randall-Selitto analgesimeter, and strain gauges held by forceps or fingers (Barrot, 2012). Thermal and mechanical nociceptive tests that access nociception following direct damage to the peripheral afferent neurons in rodents are in many ways comparable to nociceptive pain symptoms of neuropathic pain patients.

However, nociception is an unconscious process dependent on nociceptor activity, when transitioning to translational pain treatments combining cognitive and affective tests in rodents is critical. Recent experimental strategies that address cognitive and affective pain states include an experimental setting that allows the animal the choice between environments that are or not associated with painful experiences (i.e. conditioned place preference/aversion) (Sufka, 1994; Barrot, 2012). Additionally, the affective state of a rodent can be assessed by examining specific facial expression signatures (i.e. grimace scale), and a fairly novel test of vocalizations for chronic pain states (Kurejova *et al.*, 2010; Langford *et al.*, 2010).

Table 1:

Term	Definition
Pain	An unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage
Nociception	The neural processes of encoding and processing noxious stimuli
Pain threshold	The minimal intensity of a stimulus that is perceived as painful
Noxious stimulus	An actually or potentially tissue-damaging event
Nociceptive pain	Pain arising from activation of nociceptors
Neuropathic pain	Pain arising as a direct consequence of a lesion or disease affecting the somatosensory system
Allodynia	Pain in response to a non-nociceptive stimulus
Hyperalgesia	Increased pain sensitivity
Dysesthesia	An unpleasant abnormal sensation, whether spontaneous or evoked
Paresthesia	An abnormal sensation, whether spontaneous or evoked
Hypoesthesia	Decreased sensitivity to stimulation, excluding the special senses
Hyperpathia	A painful syndrome characterized by an abnormally painful reaction to a stimulus, especially a repetitive stimulus, as well as an increased threshold
Hyperesthesia	Increased sensitivity to stimulation, excluding the special senses
Peripheral sensitization	Increased responsiveness and reduced threshold of nociceptors to stimulation of their receptive field
Central sensitization	Increased responsiveness of nociceptive neurons in the central nervous system to their normal or subthreshold afferent input

Table 1: Pain terminology. (Merskey, 1994; Loeser & Treede, 2008).

2. Rodent models of peripheral neuropathic pain

Experimental rodent models of neuropathy provide the means to examine the pathological changes in neuropathic pain mechanisms. Several models have been developed to mimic different neuropathic disease states. Wall and colleagues first developed a chronic pain model by producing a sciatic nerve transection and described pathophysiological mechanisms in chronic pain that were distinct from acute noxious pain (Wall & Gutnick, 1974). From this initial model a variety of peripheral nerve injury models have been developed in animals as surrogates for neuropathic pain.

Axotomy or neuroma model is the oldest model of neuropathic pain in animals, which involves a complete transection of the sciatic nerve at mid-thigh level (Wall *et al.*, 1979a). Animals develop anesthesia dolorosa- pain is present with the absence of sensory input in that area. Since the hindpaw is completely denervated, evoked pain behavior cannot be measured. Autotomy is observed in this model, which was originally believed to occur due to spontaneous activity of C-fibers and/or excessive grooming (Wall *et al.*, 1979b; Rodin & Kruger, 1984; Devor, 1991). The major limitation of the axotomy model is that it is relatively uncommon in patients, with the exception of amputation resulting in phantom limb pain. Similar to this model is the partial nerve ligation or Seltzer model where a single ligature is made distal to where the posterior biceps-semitendinosus nerve branch off (Seltzer *et al.*, 1990). Seltzer model is less severe; therefore, tactile and thermal hyperalgesia is readily detectable. Animals

lack autotomy; however, the degree of nerve injury is not standardized and there is considerable mixing of injured and uninjured fibres (Ossipov *et al.*, 2006).

One of the most common animal models of neuropathic pain is the chronic constriction injury (CCI) (Bennett & Xie, 1988). Four loose ligatures of chromic gut are placed around the sciatic nerve proximal to the sciatic trifurcation. Behavioral changes in mechanical and thermal hyperalgesia, and cold allodynia develop within a week reaching a maximum two weeks post surgery (Bennett & Xie, 1988; De Vry *et al.*, 2004). Inflammation, intraneural edema, focal ischemia, and Wallerian degeneration occur in the constricted region of the nerve. Proximal to the constricted area, swelling is evident that most likely hinders axoplasmic transport from the soma (Bennett & Xie, 1988). CCI results in degeneration of myelinated (94%) and unmyelinated fibers (74%) within 14 days (Kajander & Bennett, 1992; Coggeshall *et al.*, 1993). However the substantial loss in primarily Aα/β fibers is less likely to account for hyperalgesia, since injury induced hyperalgesia lasts up to 56 days and Aβ-fibers remain absent. Instead, CCI-induced hyperalgesia may arise from sensitized Aδ- (lightly myelinated) and C-fibers to regenerate to near normal levels (Kajander & Bennett, 1992; Coggeshall *et al.*, 1993). The advantage to CCI is the observed pain-symptoms closely correspond to causalgia or complex regional pain syndrome and carpal tunnel syndrome in patients (Bennett & Xie, 1988). A major caveat to this model is that chromic gut sutures placed next to the sciatic nerve, instead of around the nerve, result in similar behavioral hypersensitivity (Maves *et al.*, 1993).

Chung and colleagues developed an alternative approach that involves L5 and L6 spinal nerve ligation (SNL) distal to the DRG, a variation is also performed with only L5 SNL. In this model, pain-related behavior (mechanical, thermal, and cold hypersensitivity) develops within 24hr and persists for 10-16 weeks (Kim & Chung, 1992; Choi *et al.*, 1994; LaBuda & Little, 2005). Although the surgery for this model is more complex and labor intensive, a principal advantage of this model is the ability to delineate injured and uninjured spinal nerves. Additionally, tactile hypersensitivity is robust allowing for time-dependent behavioral analysis.

The spared nerve injury (SNI) animal model of neuropathic pain involves axotomy of two of the three terminal branches of the sciatic nerve; common peroneal and tibial nerves, while leaving the sural nerve intact (Decosterd & Woolf, 2000; Shields *et al.*, 2003). Variations of SNI, include transection of the common peroneal and sural nerves, tibial and sural nerve, or each nerve separately (Lee *et al.*, 2000; Hofmann *et al.*, 2003; Brittain *et al.*, 2011), all of which display mechanical hypersensitivity within 4 days of injury that persists for several weeks. The key advantage of this model is that behavioral changes in injured and uninjured nerves may be assessed by testing corresponding receptive fields (Decosterd & Woolf, 2000). Although the innervation territories of the saphenous and sural nerve overlap, the corresponding DRG are separate. Variations of the SNI model are useful; animals display less guarding behavior with similar observations of pain hypersensitivity allowing for undisturbed behavioral assessment (Lee *et al.*, 2000; Brittain *et al.*, 2011). Overall, SNI pain-

related behavior is robust, long-lasting, and mimics many symptoms of clinical neuropathic pain.

Taken together, current models used provide unique advantages in understanding the etiology, progression and severity of neuropathic pain. Peripheral nerve damage results in pathological neural plasticity at all levels of the nociceptive pathway; sensory neurons, spinal cord, and brain in the form of peripheral and central sensitization.

E. Initial response to peripheral nerve injury

1. Acute and neurogenic inflammation

When peripheral nerve injury first occurs, there is tissue and nerve damage resulting in an acute inflammatory response by the activation of keratinocytes, mast cells, neutrophils, and macrophages (Scholz & Woolf, 2007). Immediately after injury, resident macrophages are activated and release cytokines, which recruit neutrophils (within 24hrs) and monocytes (within 48hrs) from the peripheral blood to the injury site (Mueller *et al.*, 2001; Perrin *et al.*, 2005). Activated macrophages and denervated Schwann cells secrete matrix metalloproteases that disrupt the blood-nerve barrier promoting leukocyte extravasation (Shubayev *et al.*, 2006). Vasoactive mediators, such as CGRP, substance P, bradykinin and nitric oxide are further released from injured axons resulting in hyperemia and swelling (Fried *et al.*, 1989; Zochodne & Ho, 1992; Zochodne *et al.*, 1995; Lopes & Couture, 1997; Zochodne *et al.*, 1999).

In parallel, an ‘inflammatory soup’ of pro-inflammatory mediators of bradykinins, substance P, hydrogen ions, NGF, prostaglandins, histamine, adenosine 5'-triphosphate (ATP), cytokines, and chemokines are released from damaged and immune competent cells following tissue damage or nerve injury (McMahon *et al.*, 2006). Substances released from sensory neurons can further act on surrounding cells and tissue contributing to inflammation. These bioactive substances generate the cardinal signs of inflammation, which are characterized by redness and warmth, swelling, and hypersensitivity (Richardson & Vasko, 2002; Marchand *et al.*, 2005). The redness (rubor) and heat (calor) are due to increased blood flow and body core temperature to the inflamed site resulting in neurogenic inflammation, swelling (tumor) is caused by accumulation of fluid arising from the extravasation induced by neurogenic inflammation, and pain (dulor) is due to release of sensitizing agents that stimulate nerve endings.

2. Peripheral sensitization

Peripheral sensitization is the direct result of peripheral inflammation and comprises a reduction in threshold and an increase in the excitability of the peripheral terminals of nociceptors in response to sensitizing inflammatory mediators in the injured endoneurial environment (Kessler *et al.*, 1992; Costigan *et al.*, 2009). For instance, the release of substance P into the endoneurial environment increases the excitability of small nociceptive sensory neurons (Abdulla *et al.*, 2001). Released substances, such as protons, prostaglandins, or bradykinin can also sensitize Aδ-nociceptors; however, NGF and GDNF have a

delayed nociceptive response, since both are retrogradely transported to the cell bodies of primary sensory neurons to regulate gene transcription (Fang & Luo, 1996; Leitner *et al.*, 1999; Shu & Mendell, 1999; Malin *et al.*, 2006) potentially by increasing TRPV1 expression and transduction properties (Chuang *et al.*, 2001; Shu & Mendell, 2001; Ji *et al.*, 2002), further contributing to peripheral sensitization.

Another group of sensitizing agents are cytokines, which mediate the inflammatory response between cells over short distances. Pro-inflammatory cytokines include interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF) α, while others such as IL-10 are anti-inflammatory (Moalem & Tracey, 2006). All three pro-inflammatory cytokines may sensitize nociceptors and elicit hyperalgesia (Sommer & Kress, 2004). For instance, in an *in vitro* nerve skin preparation, IL-1β was able to sensitize nociceptors by facilitating the release of CGRP and further evidence demonstrates that intraplantar injections of IL-1β were capable of eliciting hyperalgesia (Follenfant *et al.*, 1989; Fukuoka *et al.*, 1994). Moreover, topical application of TNFα to the sciatic nerve elicits ectopic activity of Aδ/C fibres and intraneural injections led to thermal hyperalgesia and mechanical allodynia (Sorkin *et al.*, 1997; Zelenka *et al.*, 2005). Although TNFα, which acts through TNFR1, can activate NaV channels, there does not appear to be direct features of the cytokine in sensitizing nociceptors (Jin & Gereau, 2006). In fact, most clinical trials of anti-TNFα therapy have been unsuccessful in alleviating neuropathic pain.

A third group of sensitizing agents is chemokines, which are chemotactic cytokines that function to recruit immune cells to damaged sites and activate GPCRs (G-protein coupled receptors). This group includes (but not limited to) monocyte chemotactic protein (MCP1), stromal derived factor (SDF)1, chemokine (C-C motif) ligand CCL3, chemokine (C-X-C motif) ligand CXCL8, and fractalkine (Abbadie, 2005; White *et al.*, 2005a). Similar to proinflammatory cytokines, chemokines may also modulate acute nociceptor activity and sensitivity (Oh *et al.*, 2001; Zhang *et al.*, 2005; Bhangoo *et al.*, 2007b). Oh and colleagues demonstrated that chemokines acting on all three families of chemokine receptors; CXCR, CCR, and CX3CR mobilized intracellular calcium (an indication of neuronal function) in DRG neurons. Further, intradermal administration of SDF1 reduced baseline evoked mechanical sensitivity (Oh *et al.*, 2001).

Additionally, pro-inflammatory cytokines and chemokines may indirectly modulate nociceptor sensitization by inducing the expression of other inflammatory mediators (Schweizer *et al.*, 1988; Nicol *et al.*, 1997a). Additional inflammatory mediators include, prostaglandin E_2 and prostaglandin I_2 that may enhance the sensitivity of primary afferents to mechanical stimuli and lower firing threshold of DRG neurons (Birrell *et al.*, 1991; Nicol *et al.*, 1997b). Taken together, initially after nerve injury peripheral sensitization alters the state of sensory neurons by lowering activation threshold for thermal and mechanical stimuli and enhancing the responsiveness of nociceptors that may lead to long lasting hyperalgesia (Sommer & Kress, 2004; Costigan *et al.*, 2009).

F. Injured sensory neuron

In addition to the initial inflammatory response to tissue damage and nerve injury, other significant interdependent processes occur in primary afferent neurons that contribute to nociceptor sensitization and the generation of a neuropathic pain state. This involves a heavily intertwined interaction with immune cells, glia cells and sensory neurons at the site of injury, the DRG soma, and in the spinal cord that in combination impact the nociceptive pain processing (Scholz & Woolf, 2007). The major events include: (1) ectopic neuronal activity in injured and intact neighboring uninjured neurons, (2) Wallerian degeneration and axonal regeneration.

1. Maladaptive ectopic activity

The sensitization of nociceptors following nerve injury changes many electrophysiological properties of primary afferent neurons. Injured sensory neurons display an increase in excitability by demonstrating a reduction in action potential threshold (Xie *et al.*, 1995; Zhang *et al.*, 1997; Zhang *et al.*, 1999; Ma & LaMotte, 2005). Nerve injury may lead to an increase in primary afferent activity in the absence of an identifiable stimulus (Study & Kral, 1996). Such spontaneous activity in nociceptors can originate from the neuroma that develops at the site of injury (Govrin-Lippmann & Devor, 1978; Liu *et al.*, 2000) and from DRG cell bodies of injured neurons (Wall & Devor, 1983; Kajander *et al.*, 1992). The DRG soma is exposed to similar endoneurial environment as in the neuroma, interestingly the DRG lacks a blood-nerve barrier and a tissue-nerve barrier

(Allen & Kiernan, 1994; Shinder & Devor, 1994; Byrod *et al.*, 2000) and is thought to have a specialized chemosensory role (Devor, 2006).

Furthermore, the ectopic activity generated or transmitted to the cell body of nociceptive sensory neurons may provide signals generating spontaneous pain (Amir *et al.*, 2005). Another interesting feature of spontaneous pain arising from nerve injury, is the ectopic activity of low-threshold large myelinated afferents (Aβ-fibers) (Campbell *et al.*, 1988). Aβ-fibers generally signal innocuous sensation, however following injury Aβ-fibers undergo a phenotypic switch and increase neuronal activity that may contribute to spontaneous pain and tactile allodynia (Tal *et al.*, 1999; Truini *et al.*, 2009; Song *et al.*, 2012; Zhu & Henry, 2012). Pharmacologically blocking Aβ-fibers prevents injury-induced tactile allodynia (Yamamoto *et al.*, 2008). Nerve injury may impact only a fraction of axons; the remaining uninjured axons are consequently exposed to the same inflammatory and degenerative products. As a result, residual neighboring intact afferent fibres also demonstrate spontaneous ectopic firing (Wu *et al.*, 2001; Wu *et al.*, 2002; Djouhri *et al.*, 2012). Taken together, nerve injury induces electrophysiological changes in injured and uninjured nociceptive and non-nociceptive DRG neurons.

Specific ion channels have been identified to contribute significantly to altered neuronal function following a nerve injury. For instance, following a nerve injury, the two-pore domain K+ channel TRESK is downregulated by 30-40% leading to a steady depolarization of the sensory neuronal membrane potential (Tulleuda *et al.*, 2011). In conjunction, subthreshold membrane potential

oscillations, largely carried by the persistent component of the sodium current, I_{NaP}, are frequently seen in injured sensory neurons and may be a major contributor to ectopic spike discharge (Kovalsky *et al.*, 2009).

Membrane potential oscillations and spontaneous activity have also been attributed to the mixed cation current I_h conducted by hyperpolarization-activated cyclic nucleotide-gated (HCN) channels. Large sensory neurons mainly express HCN1, while small sensory neurons predominantly express HCN2 (Biel *et al.*, 2009). Recently it has been demonstrated that sensory-specific HCN2 deficient animals show hardly any signs of neuropathic pain (Emery *et al.*, 2011). The current model suggests that HCN channels in concert with T-type calcium channels generate repeated cycles of depolarization and hyperpolarization, which leads to activation of I_h, contributing to the receptive firing patterns in injured neurons (Biel *et al.*, 2009).

Furthermore, voltage-gated sodium channels (VGSC) have been substantially implicated in altering neuronal activity following injury. There is evidence that NaV1.3, NaV1.6, NaV 1.7-1.9 are involved in injured and uninjured sensory neurons (Dib-Hajj *et al.*, 2010). Most notably, expressed in nearly all sensory neurons Nav1.7 plays an important role in nociception, such that gain-of-function mutations of Nav1.7 demonstrates ectopic firing of C-fibers in the absence of nerve injury and in spontaneous pain conditions (Cox *et al.*, 2006).

2. Nerve degeneration and regeneration

Within two days of injury, immune cells participate in the removal of distal degenerating axon/myelin debris and formation of a neuroma (Scholz & Woolf, 2007). Injured afferent axons distal to site of nerve injury begin to degenerate in a process referred to as Wallerian (anterograde) degeneration. At the same time, this allows for the reorganization of Schwann cells for regrowth of injured axons. Schwann cells release NGF and GDNF to promote axonal growth and remyelination (Ebadi *et al.*, 1997; Chan *et al.*, 2004).

Axonal degeneration is believed to be an intrinsic process of self-destruction that is triggered by impaired axonal transport followed by axonal fragmentation within 48 hours (Mack *et al.*, 2001; Beirowski *et al.*, 2005; Saxena & Caroni, 2007). During axonal fragmentation, Schwann cells lose axonal contact and initiate degeneration of their own myelin sheaths. The axonal and myelin debris is phagocytized by recruited macrophages. Chemokines play an essential role in early recruitment of macrophages to the site of injury (Taskinen & Roytta, 2000). For instance, the chemokine MCP1 is unregulated very early after nerve injury to participate in macrophage chemotaxis (Perrin *et al.*, 2005). In addition, many axonal growth inhibitors are released in degenerating axon/myelin sheath, thus clearance of axonal and myelin debris is critical for induction of possible axonal regeneration (Schafer *et al.*, 1996; Shen *et al.*, 1998). Moreover, the proximal segment of injured nerve is spared and remains functionally coupled to the cell body. Retrograde signals from the proximal segment induce cell body

swelling and chromatolysis (Wells & Vaidya, 1989). The proximal segment of the injured sensory neuron is now destined for survival or neuronal cell death.

Induction of axonal growth is influenced by the cytokines and neurotrophic factors present at the time of Wallerian degeneration (Golz *et al.*, 2006). For example, transgenic mice that display slow Wallerian degeneration demonstrate slow myelin clearance, deficient NGF production, differentially regulated cytokines TNFα and IL-1α/β (Brown *et al.*, 1991; Shamash *et al.*, 2002). Peripherally, collateral sprouting of intact nociceptor endings has been suggested to promote reconnection to peripheral targets (Kinnman & Aldskogius, 1986; Inbal *et al.*, 1987) and enhance sensitivity to circulating adrenaline and sympathetic activity (Sato & Perl, 1991; Rajan *et al.*, 2003).

Although regeneration occurs, a fraction of injured sensory neurons do not survive and the remainder of the proximal segment degenerates. In rats, there is a detectable decrease in small unmyelinated neurons 8 weeks after injury (Tandrup *et al.*, 2000; Okamoto *et al.*, 2001b). Whereas, in mice 24% of DRGs are lost within 7 days of nerve injury and this loss continues to increase to more than 50% by 4 weeks post injury (Shi *et al.*, 2001).

During Wallerian degeneration the surrounding inflammatory endoneurial environment (i.e. cytokines, chemokines, and growth factors) both from neuroma that develops at the site of injury and injured dorsal root ganglion may prolong peripheral sensitization of primary afferent nociceptors that may result in long lasting hyperalgesia. Overall, the injured nerve fiber produces negative signals including cell death, compromised transduction due to terminal atrophy, a

compromised conduction due to loss of peripheral axons and compromised neurotransmission due to loss of central terminals. Loss of function can manifest across the whole sensory spectrum (e.g. global numbness after a traumatic nerve injury) or it can affect specific sensory modalities.

G. Central sensitization

Following peripheral nerve injury, functional changes in primary and second order neurons are strongly believed to contribute to pain hypersensitivity associated with neuropathic pain. After a peripheral nerve injury, degeneration of C-fiber terminals occurs in Lamina II (Arvidsson *et al.*, 1986; Kapadia & LaMotte, 1987). Moreover, second order neurons in the spinal cord receive multiple inputs from A/Ç fibers and develop an increased responsiveness to their normal afferent input, a phenomenon known as central sensitization.

The concept of central sensitization was first developed by a set of fundamental experiments performed by Clifford Woolf, which demonstrated that after repeated peripheral noxious heat stimuli sufficient to generate mild inflammation of the hind paw resulted in an increased excitability of the motor neurons that lasted for several hours, and included a reduction in threshold and enlargement of the cutaneous receptive fields. Moreover, the motor neurons were now no longer nociceptive-specific but could be activated by low-threshold (innocuous) peripheral inputs by Aβ-fibers (Woolf, 1983). Overall, these experiments led to a general hypothesis that activating C-fiber nociceptors produced activity-dependent changes in the functional properties of neurons in

the dorsal horn of the spinal cord (i.e. central plasticity) capable of responding to stimuli outside of the injury area and to low-threshold afferents that previously did not activate the nociceptive system further contributing to pain hypersensitivity (Woolf, 1983; Woolf & King, 1989).

Following nerve injury, primary afferent neurons develop or increase in spontaneous activity, display reduction in threshold for activation and enlargement of nociceptive neuron receptive fields (Woolf & Salter, 2000; Devor, 2009). Spontaneous activity is likely to be an important component of neuropathic pain, because it is a major contributor to spontaneous pain and to central changes in the nociceptive pathway that amplify pain.

In neuropathic pain states central sensitization is not only initiated but maintained by altered and sensitized primary afferent neurons. The altered functional status of nociceptive and non-nociceptive neurons leads to excitability of dorsal horn neurons (Dalal *et al.*, 1999; Kohno *et al.*, 2003), heterosynaptic facilitation (Mendell & Wall, 1965; Willis, 2002; Sandkuhler, 2007), and decreases in inhibitory transmission (disinhibition) in the dorsal horn of the spinal cord(Yaksh, 1989; Sivilotti & Woolf, 1994; Baba *et al.*, 2003), contributing to many pain symptoms in neuropathic pain (Campbell & Meyer, 2006; Latremoliere & Woolf, 2009) **(Figure 3)**.

Heterosynaptic facilitation is particularly prominent in central sensitization and means that a nociceptor-specific conditioning input can enable subsequent long lasting facilitation of responses to inputs from low-threshold A/C fibers. This occurs due to changes in synaptic strength from activated to neighboring non-

activated synapses. Heterosynaptic facilitation after brief nociceptor triggering input can last for hours (Mendell & Wall, 1965; Willis, 2002; Sandkuhler, 2007).

A loss of inhibition in the dorsal horn can also play an important role in central sensitization. For example, inhibiting GABA (gamma-aminobutyric acid) and glycine in the spinal cord increases A-fiber-mediated excitatory transmission in the superficial dorsal horn and following a partial peripheral nerve injury can result in a reduction of GABA-induced IPSCs in the dorsal horn (Sivilotti & Woolf, 1994; Baba *et al.*, 2003). Moreover, it is strongly believed that central sensitization is in part mediated by the interactions of non-neuronal cells (i.e. microglia and astrocytes) and nociceptive neurons (Garrison *et al.*, 1991; Colburn *et al.*, 1997). Sensitized nociceptive neurons release a host of substances in the dorsal horn, including substance P, glutamate, CGRP, ATP, and fractalkine, that may activate astrocytes and microglia in the spinal cord (Watkins & Maier, 2002). Taken together, central sensitization provides a mechanistic explanation for how low threshold A- or C-fibers can begin to produce pain, why there is a spread of sensitivity beyond areas of tissue injury or outside a damaged nerve territory, why repeated stimuli at a fixed intensity can lead to a progressive increase in pain, and why pain may long outlast a peripheral stimulus.

Figure 3:

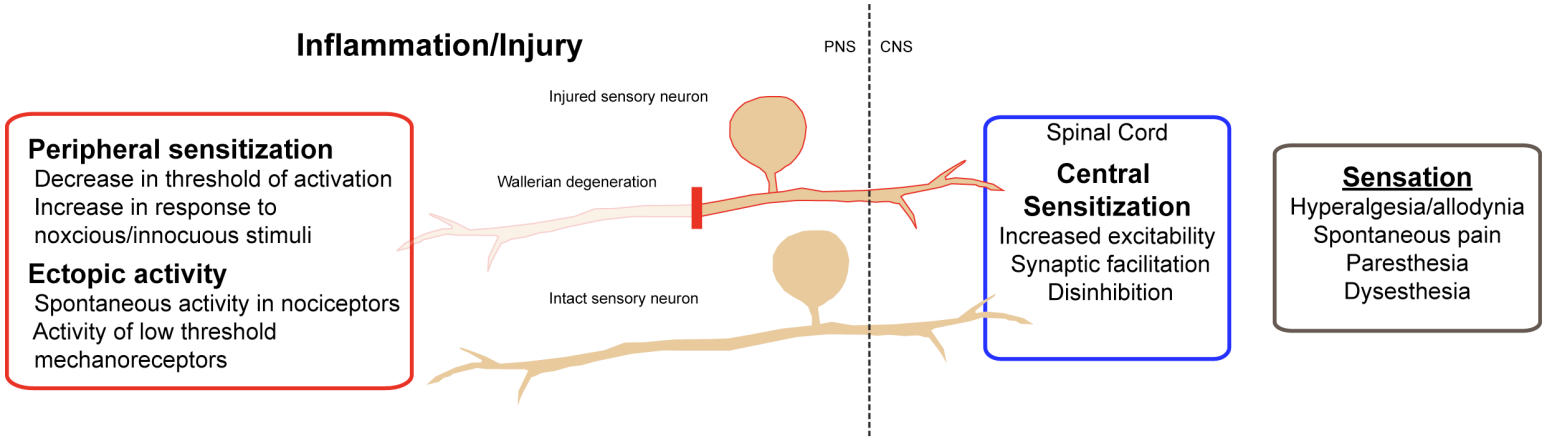


Figure 3: Overview of maladaptive changes that occur after peripheral nerve injury. In the peripheral nervous system, injury leads to the sensitization of nociceptors and ectopic activity of primary afferent neurons. In the central nervous system, altered activity of primary afferent neurons leads to central sensitization of dorsal horn neurons and may present as hyperalgesia, allodynia, spontaneous pain, paresthesia, or dysesthesia. Adapted from (Devor, 2006; Costigan *et al.*, 2009)

H. Transition from nerve injury to neuropathic pain

Only a fraction of patients with nerve damage develop neuropathic pain. For instance, sectioning the sciatic nerve or multiple intercostal nerves during thoracotomy produces neuropathic pain in 30-60% of patients (Maguire *et al.*, 2006; Ketz, 2008). Understanding the differences that underlie the susceptibility to neuropathic pain is critical in deciphering the progression to a pathophysiological neuropathic pain state. At the forefront, a genetic predisposition may serve to enhance susceptibility to the development of neuropathic pain. Recent findings have identified several genetic polymorphisms that are associated with neuropathic pain states (Lacroix-Fralish & Mogil, 2009). Polymorphisms in TRP channels, CACNG2- a trafficking protein of glutamatergic AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors, and the potassium subunit KCNS1 have been suggested as potential genetic indicators of chronic pain (Costigan *et al.*, 2010; Nissenbaum *et al.*, 2010; Binder *et al.*, 2011). It is equally important to discern phenotypical changes in the nociceptive pathway that contribute to the progression of an irreversible neuropathic pain state.

Compared to inflammatory pain, neuropathic pain exhibits a substantial change in gene expression in injured and uninjured neurons that alter membrane properties, growth, and neurotransmitter function (Costigan *et al.*, 2002; Xiao *et al.*, 2002; Obata *et al.*, 2003; Rodriguez Parkitna *et al.*, 2006). For instance, several ion channels have been identified to contribute significantly to signal transmission and neuronal excitability along the pain axis, including Nav1.3,

Nav1.7-1.9 (Dib-Hajj *et al.*, 2010). Reduction in potassium channels (Kim *et al.*, 2002; Chien *et al.*, 2007) and auxiliary subunits of voltage-gated calcium channels (Luo *et al.*, 2001; Newton *et al.*, 2001) are also evident after peripheral nerve injuries.

After injury, sensory neurons display a phenotypic switch (Decosterd *et al.*, 2002) that may involve differences in gene expression and signals from the injured environment (Wu *et al.*, 2002). For instance, CGRP, and substance P are downregulated in injured sensory neurons (Marchand *et al.*, 1994; Nahin *et al.*, 1994; Sommer & Myers, 1995; Hofmann *et al.*, 2003), where as large sensory neurons begin to express and respond to substance P (Baranowski *et al.*, 1993; Noguchi *et al.*, 1995), such that substance P receptor antagonists (NK-1 receptor) can attenuate or delay the onset of pain-related behaviors in response to nerve injury (Jang *et al.*, 2004). Another example is GDNF family receptor components (GFα1-4), GFα2 is reduced in in small diameter DRG neurons, where as GFα3 is increased in small diameter DRG neurons after injury, such changes may influence the regenerative properties of sensory neurons and response to neurotrophic factors after injury (Bennett *et al.*, 2000; Wang *et al.*, 2011a). Many of these changes in gene expression are under the control of master transcriptional regulators such as Sox11, c-Jun, and ATF3 (Lindwall & Kanje, 2005; Jankowski *et al.*, 2006; Seijffers *et al.*, 2007; Hunt *et al.*, 2012). Global changes in gene expression may further contribute to the presence of pro-nociceptive mediators in the injured extracellular environment.

Several pro-inflammatory mediators have been implicated in neuropathic pain; bradykinin, ATP, serotonin, eicosanoids, neurotrophins, reaction oxygen species (ROS), and cytokines (Moalem & Tracey, 2006). For instance, NGF antagonism at the site of nerve injury may attenuate or delay the onset of hyperalgesia after chronic compression injury (Herzberg *et al.*, 1997; Ro *et al.*, 1999). In previous work by White and colleagues, following demyelinated injury model of neuropathic pain, primary sensory neurons were directly sensitized by chemokine MCP1 and continued to express C-C chemokine receptor type -CCR2, a receptor for chemokine MCP1 (White *et al.*, 2005b; Jung *et al.*, 2009). An additional example is the cytokine IL-1β, which is unregulated in the sciatic nerve following nerve injury, and intrathecal administration of neutralizing antibodies to IL-1R can alleviate nerve injury-induced pain behavior (Gillen *et al.*, 1998; Sommer *et al.*, 1999; Okamoto *et al.*, 2001a). Recently, long-term exposure to IL-1β has been shown to increase the excitability of small and medium non-peptidergic sensory neurons (Stemkowski & Smith, 2012). Thus, cytokines play an integral role in sensitizing primary sensory neurons possibly by transactivation of TRP channels (Bandell *et al.*, 2004; Jung *et al.*, 2008; Ruparel *et al.*, 2008).

Moreover following a peripheral nerve injury, primary afferent synaptic transmission and glia activation in the spinal cord also promotes induction of proinflammatory cytokines (i.e. IL-1β, IL-6, IL-10, and TNFα) that can directly act on central terminals of primary sensory afferents and alter the function of second order neurons in the dorsal horn (Watkins & Maier, 2002; Tsuda *et al.*, 2005; Beggs & Salter, 2007). Taken together, when inflammation subsides and

neuropathic pain persists many of these pro-nociceptive mediators may continue to impact nociceptive signaling in sensory neurons and at the level of the spinal cord. A better understanding of which extracellular pro-nociceptor mediators and cytokines present (i.e. post inflammatory resolution) can aid in understanding progressive changes in sensory neurons that lead to neuropathic pain.

I. Maintenance of pro-nociceptive cytokines

Very little is known in regards to the maintenance of cytokine networks and the progression of neuropathic pain (Sommer & Kress, 2004). A prime candidate is a class of endogenous molecules, known as alarmins that signal tissue and cell damage (Yang & Oppenheim, 2004; Oppenheim & Yang, 2005). Alarmins belong to a family of molecules known as damage associated molecular patterns (DAMPs), which also include exogenous molecules- pathogen associated molecular pattern (PAMPs) that can trigger the rapid activation of the immune system and production of pro-inflammatory cytokines (Oppenheim & Yang, 2005; Bianchi, 2007). Several distinguishing characteristics of alarmins include: (1) rapidly released following non-programmed cell death, (2) cells may produce and release alarmins without dying, (3) recruit and activate cells thereby directly or indirectly promote cellular responses, (4) restore homeostasis by promoting reconstruction of tissue and resolution of inflammation (Bianchi, 2007). Alarmins are structurally diverse multifunctional molecules that include but not limited to S100 proteins, HSPs, defensins, IL-1α, and high mobility group box 1 (HMGB1) (Oppenheim & Yang, 2005; Chan *et al.*, 2012). Some of the alarmins

engage classical receptors leading to inflammatory and immune response, TLRs and IL-1R. RAGE is another prominent receptor for some S100s and HMBG1. Collectively, alarmins interaction with TLRs, IL-1R and RAGE lead to NFκB activation, suggesting overlapping downstream signaling pathways (Bianchi, 2007; Sakaguchi *et al.*, 2011).

At one level alarmins have beneficial qualities; such as, facilitating wound repair (Straino *et al.*, 2008; Hirsch *et al.*, 2009; Zhang *et al.*, 2012). For instance, topical application of HMGB1 to wounds of diabetic mice enhanced arteriole density, granulation tissue deposition, and enhanced wound healing. Interestingly, diabetic mice at baseline display a lower level of endogenous HMGB1 compared to normal mice (Straino *et al.*, 2008). However, excessive release of alarmins contributes to a host of inflammatory, autoimmune conditions, and cancers (Sims *et al.*, 2010). For instance, the release of extracellular S100 proteins by chrondrocytes results in increased production of matrix-degrading enzymes leading to cartilage degradation and further development of arthritis (Yammani, 2012). In some cases, S100B protein has a dose-dependent effect, at a low dose S100B promotes neurite outgrowth and at a high dose leads to neuronal apoptosis (Huttunen *et al.*, 2000). It has been hypothesized that in settings in which alarmins may accumulate in microenvironments, both by failure of clearance or perpetuation of signals eliciting their release, tips the biological balance to favor the development of chronic disease (Ramasamy *et al.*, 2011).

Taken together, the relationship between the level of alarmins and degree of disease pathology appear to coincide. It is unclear in pathologies such as

neuropathic pain, which involve both beneficial (i.e. axonal regeneration) and detrimental processing (i.e. peripheral/central sensitization), whether the chronic presence of alarmin-induced events maintains altered cytokine profiles contributing to maladaptive nociceptive signaling.

J. Unconventional cytokine: High mobility group box 1

HMGB1 was discovered and identified as a nuclear factor to enhance DNA transcription (Goodwin *et al.*, 1973; Goodwin *et al.*, 1975). HMGB1 was rediscovered under a different name p30 or amphoterin, while studying neuronal development and neurite outgrowth (Daston & Ratner, 1991; Merenmies *et al.*, 1991). HMGB1 has also been called HMG-1, p30, sulphoglucuronyl carbohydrate binding protein-1 (SBP1) and differentiation enhancing factor (DEF) (Huttunen & Rauvala, 2004). The nomenclature was revised to HMGB1 (Bustin, 2001).

HMGB1 belongs to a family of non-histone chromosomal proteins, including HMGB2-4 (Stros *et al.*, 2007). Expressed as a single polypeptide chain of 215 amino acids, HMGB1 contains two N-terminal DNA binding domains HMG Box A and Box B, and an acidic C-terminal domain. Interestingly, HMGB1 lacks an endoplasmic reticulum (ER) localization sequences, but instead had two nuclear localization sequences (NLS) **(Figure 4)** (Read *et al.*, 1993; Weir *et al.*, 1993; Hardman *et al.*, 1995; Bonaldi *et al.*, 2003). Inside the cell, HMGB1 can bind to DNA regulating transcription and chromosomal architecture.

About 25 years after HMGB1 was discovered, Tracey and colleagues determined that HMGB1 is released from primary monocytes and functions as a

critical cytokine to mediate the immune response to infection and injury (Wang *et al.*, 1999). HMGB1 can be released from monocytes, tissue macrophages, astrocytes, microglia, and neurons (Passalacqua *et al.*, 1998; Wang *et al.*, 1999; Faraco *et al.*, 2007; Kim *et al.*, 2008; Gao *et al.*, 2011). In this way, HMGB1 orchestrates different cellular functions in consequence to environmental and homeostatic cues to act as a signal for tissue damage, injury and/or infection.

Figure 4:

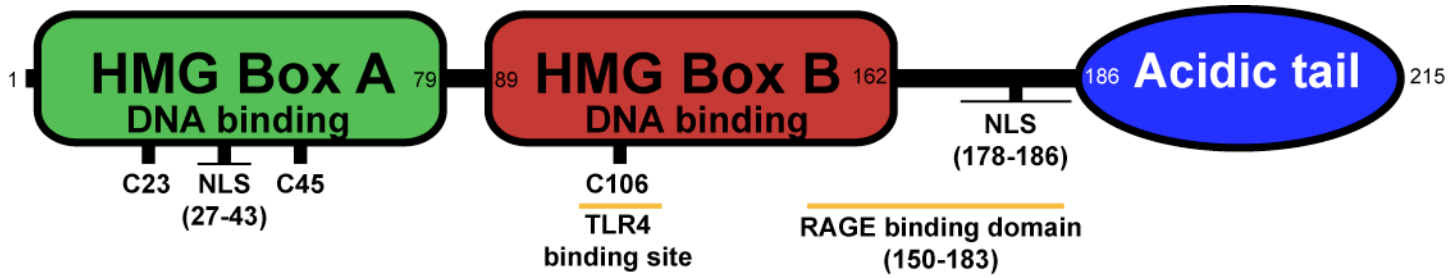


Figure 4: Schematic of High mobility group box 1 protein. HMGB1 is composed of two DNA binding domains, HMG Box A, HMG Box B, and an acidic tail region. Intracellular shuttling of HMGB1 between the cytoplasm and nucleus is regulated by post-translational modifications of lysine residues in the nuclear localization sequence (NLS). Truncation of the full length HMGB1 demonstrates that the extracellular cytokine activity resides with the Box B domain. The cysteine (C) in position 106 in the Box B domain is critical for its cytokine role, such that oxidation or selective mutation of C106 abolishes the activity of HMGB1 signaling to active cytokine release. The Box A domain can competitively inhibit the binding of HMGB1 to its receptors and attenuate the proinflammatory effect of HMGB1 and the Box B domain. Box A is considered a specific competitive antagonist of HMGB1. C106 is necessary for HMGB1 to bind to TLR4. A disulfide bond between C23 and C45 is also required for interaction of HMGB1 with TLR4. HMGB1 residues 150-183 interact with RAGE. Adapted from (Rauvala & Rouhiainen, 2010).

1. Function in DNA transcription and recombination

As a nuclear molecule, HMGB1 contains two N-terminal DNA binding domains HMG Box A and B that demonstrate nonspecific interaction with DNA and transcription factors to alter chromosomal architecture. Specifically, the Box A and B regions of HMGB1 bind to linker DNA bulges between nucleosomes and unfold chromatin structure to alter DNA transcription (Bianchi *et al.*, 1989; Hardman *et al.*, 1995; Saito *et al.*, 1999; Gaillard & Strauss, 2000). Animals that lack HMGB1 have impaired transcriptional control of the glucocorticoid receptor and die within 24hrs after birth due to hypoglycemia (Calogero *et al.*, 1999). Moreover, HMGB1 may also regulate transcription of viral promoters, including adeno-assoicated virus, herpes virus, and human immunodeficiency virus (Costello *et al.*, 1997; Naghavi *et al.*, 2003; Song *et al.*, 2004).

HMGB1 is known to interaction with transcription factors and chromatin, although this interaction is generally transient or unstable (Ueda & Yoshida, 2010). For instance, HMGB1 interacts with numerous transcriptional regulators, such as p53, HOX proteins, Rel, NFAT2, and PU.1 to facilitate expression or repression of targeted genes (Zappavigna *et al.*, 1996; McKinney & Prives, 2002; Agresti *et al.*, 2003; Mouri *et al.*, 2008; Liu *et al.*, 2009). For instance, recent evidence demonstrates that HMGB1 may bind to TNFα promoter and promotes the transcriptional repressor RelB, which is the repressive form of NFκB protein, aiding in endotoxin tolerance (El Gazzar *et al.*, 2009). Furthermore, there is substantial evidence demonstrating that HMGB1 and histone H1, a chromatin-

binding protein, compete for binding sites on chromatin further regulating transcriptional activation (Ogawa *et al.*, 1995; Catez *et al.*, 2004; Ju *et al.*, 2006).

HMGB1 is also involved in V(D)J recombination during early stages of immunoglobulin T-cell receptor production (Stros, 2010), which enables the immune system to recognize and adapt to new pathogens. Specifically, HMGB1 stimulates the RAG-RSS complex to initiate V(D)J recombination by introducing a double stranded cleavage (Agrawal & Schatz, 1997; Kwon *et al.*, 1998). Collectively, HMGB1 can interact directly with DNA, chromatin, and transcription factors to regulate transcription and genetic recombination.

2. Functions as a cytokine through active and passive release

Through a series of truncation experiments, the proinflammatory activity of HMGB1 was determined to reside in the Box B domain **(Figure 4)**. The first 20 amino acid residues (position 89-108) represent the minimal peptide sequence that retains cytokine-inducing activity (Li *et al.*, 2003). Although Box A has a 40% sequence homology to Box B, Box A lacks proinflammatory activity. Instead, the Box A domain acts as an antagonist to Box B, where it competes for HMGB1 binding sites preventing the induction of pro-inflammatory mediators TNFα and IL-1β (Yang *et al.*, 2004) **(Figure 4)**.

During tissue damage, infection, injury, or by other inflammatory stimuli, HMGB1 is released and mediates systemic inflammatory responses in immune cells and endothelial cells to produce proinflammatory mediators, such as TNFα, IL-1β, and Interferon (IFN)-γ (Andersson *et al.*, 2000; Bianchi, 2007). As a

cytokine, HMGB1 is not limited to immune cells but is also released from neuronal and non-neuronal cell types following tissue damage or injury (Kim *et al.*, 2006; Faraco *et al.*, 2007). The manner in which HMGB1 is released is through active or passive secretion from the nucleus into the extracellular space.

Proinflammatory mediators and endotoxin- lipopolysaccharide (LPS) can promote the active secretion of HMGB1 (Wang *et al.*, 1999). The translocation of nuclear HMGB1 into the cytosol is facilitated by the acetylation on one of its nuclear localization signals, which prevents re-entry into the nucleus (Bonaldi *et al.*, 2003). In macrophages, acetylated HMGB1 migrates to cytoplasmic secretory lysosomes for subsequent release into the extracellular space (Gardella *et al.*, 2002a). Nucleocytoplasmic shuttling of HMGB1 is further regulated by protein kinase C (PKC) or by calcium/calmodulin-dependent protein kinase (CaMK) IV phosphorylation of HMGB1 (Youn & Shin, 2006; Zhang *et al.*, 2008; Oh *et al.*, 2009).

For those cell types that do not contain secretory lysosomes (i.e. neurons), active HMGB1 secretion may involve non-vesicular translocation from the cytoplasm to the plasma membrane (Nickel & Rabouille, 2009). Additionally, HMGB1 secretion may be mediated by inflammasome assembly, which is cytosolic multiprotein complex that proteolytically activates caspase-1 to facilitate the release of cytokines, such as IL-1β and IL-18 (Lamkanfi *et al.*, 2010). However, not all cell types undergo active section of HMGB1. LPS stimulation in kidney cells, adrenal cells, and primary T cells does not facilitate release of HMGB1 (Wang *et al.*, 1999).

On the other hand, necrotic inflammatory cells, neurons and astrocytes undergo passive secretion of HMGB1, where loosely bound nuclear HMGB1 rapidly diffuses across a permeabilized plasma membrane (Giancotti *et al.*, 1996; Scaffidi *et al.*, 2002; Tsung *et al.*, 2007; Kim *et al.*, 2008). For instance in neutrophils, post-translational mono-methylation of HMGB1 or poly (ADP)-riboslylation alters its DNA binding activity resulting in redistribution of HMGB1 by passive diffusion out of the nucleus (Giancotti *et al.*, 1996; Ditsworth *et al.*, 2007; Ito *et al.*, 2007). Whether through active or passive cytoplasmic and extracellular release, HMGB1 is a versatile cytokine responding to its environment.

3. Extracellular HMGB1

Extracellular HMGB1 may form a complex with other molecules to enhance proinflammatory responses, including LPS, IL-1, bacterial DNA, CXCL12, CD24, and viral RNA (Sha *et al.*, 2008; Campana *et al.*, 2009; Chen *et al.*, 2009; Hreggvidsdottir *et al.*, 2011). Interestingly in some cases, application of recombinant HMGB1 alone was shown to lack cytokine function (Tsan, 2011). Molecular properties of three cysteine residues have been implicated to be involved in HMGB1 cytokine activity; the redox state of cysteine (C) 106, and a disulfide bond between C23 and C45 (Hoppe *et al.*, 2006; Sahu *et al.*, 2008). In mild oxidative conditions an intramolecular disulfide bond is formed between C23 and C45 in HMG Box A domain, while C106 remains redox-inactive (Hoppe *et al.*, 2006; Sahu *et al.*, 2008). Both disulfide and reduced HMGB1 have been detected in supernatants following LPS treatment in THP-1 (human acute monocytic

leukemia cell line) cells (Venereau *et al.*, 2012). Moreover, Schiraldi and colleagues demonstrated that the formation of a completely reduced HMGB1-CXCL12 (all-thiol-HMGB1) heterocomplex (Schiraldi *et al.*, 2012) is necessary for monocyte recruitment, compared to disulfide-HMGB1 (Venereau *et al.*, 2012). Extracellular HMGB1 has chemotactic qualities to recruit enterocytes, smooth muscle, and endothelial, and stem cells (Degryse *et al.*, 2001; Sappington *et al.*, 2002; Palumbo *et al.*, 2004; Mitola *et al.*, 2006; Yang *et al.*, 2007; Vezzoli *et al.*, 2010).

During inflammation the predominant form of HMGB1 is C106 thiol (reduced) and disulfide bond C23 and C45 (disulfide-HMGB1); however, when inflammation begins to subside, HMGB1 is terminally oxidized cysteine residues rendering its biological activity (oxidized-HMGB1) (Antoine *et al.*, 2010; Vezzoli *et al.*, 2010; Yang *et al.*, 2011). An oxidizing environment following inflammation or injury may promote HMGB1 cytokine activity, instead of cellular repair by monocyte recruitment (Vezzoli *et al.*, 2011). **Table 2** provides a summary of HMGB1 redox states and associated biological activity.

Less is known about HMGB1-mediated degradation. Extracellular HMGB1 undergoes limited proteolysis by serine proteinases that are secreted by stimulated cells. Interesting, the degradation of HMGB1 can produce a 10 amino acid fragment that retains some functional activity in erythroleukaemia cells (Sparatore *et al.*, 2001). Further investigation is needed to determine the biological properties of extracellular HMGB1.

4. Receptor activation and downstream signaling

Extracellular HMGB1 transduces cellular signals through plasma membrane receptors, Toll like receptor (TLR) 2,4,9, and receptor for advanced glycation end products (RAGE) (Hori *et al.*, 1995; Park *et al.*, 2004; Ivanov *et al.*, 2007). Specific HMGB1 receptor interaction has been identified for RAGE and TLR4, COOH-terminal motif and cysteine (C) 106 amino acid, respectively (Huttunen *et al.*, 2002; Yang *et al.*, 2010). Within the HMG Box B domain, the RAGE binding domain has been mapped comprising amino acids position 150-183 (Huttunen *et al.*, 2002). Additionally, the reduced form of the amino acid residue C106 in the Box B domain and a disulfide bond between C23 and C45 is required for the binding of HMGB1 and TLR4 (Yang *et al.*, 2010; Yang *et al.*, 2011) **(Table 2)**. The disulfide bond between C23 and C45 further increases the stability of the folded full length HMGB1 molecule (Sahu *et al.*, 2008).

Downstream signaling of HMGB1 receptor activation is facilitated by a number of adaptor proteins (Sakaguchi *et al.*, 2011), which converge through pathways involving MAPK and NFκB, transcriptional regulator p53 (Palumbo *et al.*, 2007; Palumbo *et al.*, 2009; Penzo *et al.*, 2010), and further results in the production and release of proinflammatory cytokines, TNFα, IL-1, IL-6, IL-8, and several chemokines (Andersson *et al.*, 2000; Park *et al.*, 2003; Pedrazzi *et al.*, 2007; Wu *et al.*, 2010b; Ren *et al.*, 2012). In doing so, HMGB1 produces a broad repertoire of immunological and cellular responses that may involve cytokine production, cell proliferation, chemotaxis, angiogenesis, and cell differentiation.

Table 2:

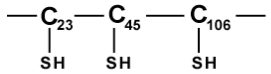
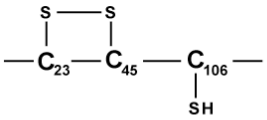
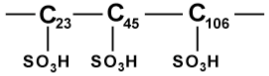
Cysteine redox level	Schematic molecular overview	Cytokine activity	Chemoattractant activity	Binding/Function	References
REDUCED- HMGB1 (all- thiol)		No	Yes	Heterocomplex with CXCL12 Preferentially interacts with RAGE induces toxicity in cancer cells	Schiraldi, 2012 Venereau, 2012 Campana, 2009 Tang, 2010a,b Vezzoli, 2010
DISULFIDE-HMGB1		Yes	No	Preferential binding to TLR4 Dominant form during inflammation	Yang, 2011
OXIDIZED-HMGB1 (terminal)		No	No	Dominant form during resolution of inflammation	Antoine, 2010 Yang, 2011

Table 2: HMGB1 redox states and biological activity. Adapted from (Venereau *et al.*, 2012).

5. Central nervous system

In early nervous system development, HMGB1 functions in neurite outgrowth, and neuronal migration and differentiation (Rauvala & Pihlaskari, 1987; Merenmies *et al.*, 1991; Fages *et al.*, 2000). During early cortical development, HMGB1 is highly expressed in migrating neurons of the cortical plate and the ventricular zone of the cerebral cortex (Nair *et al.*, 1998; Zhao *et al.*, 2000; Chou *et al.*, 2004). For instance, during early development (E16), HMGB1 is localized primarily to the cytoplasm and extracellular space of the cortical subplate of the developing primary cortex (Guazzi *et al.*, 2003). Moreover, *in vitro* functional studies have demonstrated that the interaction of extracellular HMGB1 and RAGE promotes neurite extension in embryonic rat neurons (Rauvala & Pihlaskari, 1987; Merenmies *et al.*, 1991; Huttunen *et al.*, 2000; Zhao *et al.*, 2011).

In CNS injuries and diseases, HMGB1 is most likely released locally and may function as a neuroinflammatory factor contributing to neuronal death, microglia activation, and neuronal degeneration (Kim *et al.*, 2006; Faraco *et al.*, 2007; Gao *et al.*, 2011; Kim *et al.*, 2011). In animal models of cerebral ischemia, early HMGB1 release is evident in damaged neurons and astrocytes (Kim *et al.*, 2006; Pedrazzi *et al.*, 2007; Kim *et al.*, 2008; Muhammad *et al.*, 2008; Qiu *et al.*, 2008), and injections of anti-HMGB1 antibody 6 hours after focal cerebral ischemia is neuroprotective (Liu *et al.*, 2007). The disruption in the blood-brain barrier in CNS ischemia models allows HMGB1 to enter the blood stream (Parkkinen & Rauvala, 1991; Qiu *et al.*, 2010). Following spinal cord ischemia a

decrease in HMGB1 serum levels is associated with better motor neuron survival (Wang *et al.*, 2009; Gong *et al.*, 2012). During the recovery phase after stroke, astrocytes may produce HMGB1 to facilitate neurovascular repair (Hayakawa *et al.*, 2010a; Hayakawa *et al.*, 2010b). Finally after spinal cord compression injury, HMGB1 is elevated in the spinal cord tissue of rodents and further associated with neuronal apoptosis (Kawabata *et al.*, 2010; Chen *et al.*, 2011). Recently, it has been proposed that HMGB1 may pose as an effective therapeutic target for spinal cord injury repair (Kikuchi *et al.*, 2011).

Extracellular HMGB1 may also have a critical role secondary inflammation by activating microglia activation and secondary infiltration of immune cells. For instance, HMGB1 can rapidly activate microglia and is present in microglia for up to 4 days after cerebral ischemia (Kim *et al.*, 2006; Kim *et al.*, 2008). HMGB1 was found to bind Mac1, a microglial membrane receptor, activating NFκB signaling resulting in progressive inflammation and dopaminergic neurodegeneration (Gao *et al.*, 2011). Neutralizing HMGB1 antibody was able to block progressive neurodegeneration, suggesting a novel mechanism for chronic Parkinson's disease progression (Gao *et al.*, 2011).

HMGB1 functions in other neurological diseases; such as, Alzheimer's disease, Huntington's disease, Multiple sclerosis, and amyotrophic lateral sclerosis (ALS). In ALS, degenerating motor neurons display a decrease in immunostaining for HMGB1, whereas in the spinal cord HMGB1 was increased in reactive microglia in the spinal cord with ALS (Lo Coco *et al.*, 2007; Casula *et al.*, 2011). Interestingly, in Alzheimer's disease HMGB1 acts as a decoy by

stabilizing Aβ42 oligmers and prevents microglial phagocytosis of Aβ42 oligmers (Takata *et al.*, 2003). Additionally, elevated levels of HMGB1 in the brain induce memory abnormalities by either RAGE or TLR4 (Mazarati *et al.*, 2011), even acute application of HMGB1 has been shown to impair long-term potentiation (LTP) (Costello *et al.*, 2011). Collectively, increased HMGB1 expression and signaling may be the primary cause of cognitive and synaptic dysfunction (Costello *et al.*, 2011).

Recent evidence suggests that HMGB1 has the ability to promote seizure activity. Compelling evidence from Maroso and colleagues demonstrates that administration of HMGB1 exacerbates seizure activity induced by kainic acid. It was further shown that HMGB1 antagonists could dramatically reduce spontaneous seizures in epileptic mice for several hours, suggesting a novel mechanism for how spontaneous seizures may arise endogenously (Maroso *et al.*, 2010). The authors further hypothesized that the constitutive signaling of HMGB1 activates the ionotropic glutamate N-methyl-D-aspartate receptor (NMDAR). There results demonstrate that injections of ifenprodil, a selective blocker of NR2B subunit of the NMDA receptor, effectively eliminated the proconvulsant effects of HMGB1. In fact administration of HMGB1 alone can increase NR2B phosphorylation (Maroso *et al.*, 2010), and most recently HMGB1 was found to co-immunoprecipitate with NR2B (Pedrazzi *et al.*, 2012). From these recent findings, HMGB1 may contribute to enhanced neuronal excitability and likely disrupts neurophysiological function that may contribute to CNS disorders/diseases.

6. HMGB1 and pain

Similar to other cytokines, HMGB1 may contribute to acute and chronic pain states. A number of studies demonstrate peri-sciatic or intrathecal administration of HMGB1 produces rapid and transient mechanical allodynia and thermal hyperalgesia in rodents (Chacur *et al.*, 2001; O'Connor *et al.*, 2003; Shibasaki *et al.*, 2010). A high dose of HMGB1 can even produce bilateral mechanical allodynia suggesting its potency and indirect nature of inducing pain states (Chacur *et al.*, 2001). In animals models of diabetic pain, lower back pain, and bone cancer pain, neutralizing HMGB1 antibodies can effectively reverse disease-induced pain behavior outcomes (Tong *et al.*, 2010; Otoshi *et al.*, 2011b; Ren *et al.*, 2012).

A recent study by Shibasaki and colleagues demonstrate that administration of HMGB1 neutralizing antibody prior to spinal nerve ligation and continuous infusion thereafter can prevent the development of neuropathic pain (Shibasaki *et al.*, 2010). Within the first week following injury, there was an increase of cytoplasmic HMGB1 and RAGE in Schwann cells, injured DRGs and satellite glial cells (SGCs) (Shibasaki *et al.*, 2010). Moreover, after sciatic nerve transection, HMGB1 is increased at the distal point of transection and appears to mediate the neuron-Schwann cell interaction in regenerating peripheral nerves (Daston & Ratner, 1991). Additional research is needed to determine whether HMGB1 influences the function of primary afferent neurons in different chronic pain states. **Table 3** provides a summary of relevant rodent pain behavior studies examining HMGB1.

Table 3:

Injury	Animal	Route of Administration	Mechanical Allodynia	Thermal Hyperalgesia	Reference
Naïve	S/D rats	Perisciatic nerve, HMG (0.5µg)	Pain, .5-24hrs after ipsilateral	N/A	Chacur, 2001
Naïve	S/D rats	Perisciatic nerve, HMG (3.0 µg)	Pain, 1-24 hr both sides	N/A	Chacur, 2001
Naïve	S/D rats	Perisciatic nerve, HMGB1 (0.001-0.1mg/kg)	Pain, 1-24 hr after injection	Pain, 1-24 hr after injection	Shibasaki, 2010
Naïve	S/D rats	Perisciatic nerve, HMGB1 (0.1mg/ml) + anti-HMGB1 (.04µg/kg)	Reversed	Reversed	Shibasaki, 2010
Naïve	S/D rats	i.t. HMGB1 (1000ng)	Pain, 40-120 min after injection	N/A	O'Connor, 2003
Bone cancer pain	Wistar Rats	i.t. ant-HMGB1 (Box B)	Reversed 1-24hr, PID 15	N/A	Tong, 2011
L5 SNL	S/D rats	i.t. anti-HMGB1 antibody (6.8mg/kg) catheter at L5 ligation site, before injury , followed by infusion 7X (1.6mg/kg)	Reversed, PID 3-7	No effect	Shibasaki, 2010
Autologous Nucleus Pulposus Onto Nerve roots in rats	S/D rats	i.p. anti-HMGB1 (400 µg) after surgery	Reversed, PID 2-14	N/A	Otoshi, 2011
Diabetic pain (Type 2 diabetes)	db/db mice	i.t. anti-HMGB1(Box B)	Reversed, postnatal 1-5 months	N/A	Ren, 2012

Table 3: Rodent pain behavior studies examining HMGB1.

K. Toll-like receptors

Toll-like receptors (TLRs) are an evolutionally conserved class of PAMP receptors that function to sense and initiate host defense mechanisms (Medzhitov *et al.*, 1997). Although the first description of the toll protein in Drosophila related to its function in dorsoventral polarity in developing Drosophila embryos, it was later discovered that the Toll ligand Spatzle was essential for the antifungal innate immune response (Anderson *et al.*, 1985a; Anderson *et al.*, 1985b; Lemaitre *et al.*, 1996). Thirteen TLRs have been identified in mammals so far that recognize a myriad of unrelated exogenous and endogenous molecules, including bacteria derived endotoxins or pathogen-specific nucleic acids. TLRs are involved in wide range of biological processes from pathogen recognition to mediating cellular repair and injury (Gangloff *et al.*, 2003).

The structure of TLRs are representative of type I integral membrane proteins that have three distinct regions; the extracellular domain, transmembrane domain, and intracellular domain. Ligand interaction occurs at the extracellular domain consisting of leucine rich repeat motifs, which facilitates the hetero- and homodimerization of TLRs. Within the intracellular domain, TLRs contain a conserved ~200 amino acid region, known as the toll/interleukin 1 receptor (TIR) domain (Slack *et al.*, 2000). The TIR domain is conserved among TLRs and the IL-1R, which mediates protein-protein interactions between TIR domain and adaptor proteins (Dunne & O'Neill, 2003). One common adaptor of TLRs and IL-1R is myeloid differentiation primary response protein 88 (MyD88), which shares similar downstream signaling of NFκB and MAPK activation (Muzio

et al., 1997; Adachi *et al.*, 1998; Kawai *et al.*, 1999). Interestingly, evidence suggests that adaptor protein binding is influenced by the dimerization and cellular localization of TLRs. The nucleic acid-sensing TLRs (TLR3, TLR7, TLR8 and TLR9) are localized and signal in endosomes. The compartmentalization of nucleic acid sensing TLRs is important because of their sensitivity to host DNA, which can trigger the development of autoimmune diseases if exposed to it. Host DNA is usually excluded from endolysosomes; where as, other nucleic acids from viruses are typically localized.

TLR downstream signaling has been identified by the adaptor proteins recruited to TIR domain and the subsequent gene expression profile. The TLR intracellular TIR domain can recruit TIR containing intracellular adaptor proteins to mediate signaling; MyD88, TIR domain-containing adapter protein (TIRAP; also known as Mal), TIR domain-containing adapter inducing interferon-β (TRIF), or TRIF-related adaptor molecule (TRAM). Typically, ligand specific MyD88- and TRIF-dependent pathways characterize TLR activation (Sakaguchi *et al.*, 2011). With exception of TLR3, all other TLRs can signal through MyD88-dependent pathways (Hoebe *et al.*, 2003).

The MyD88-dependent pathway results in the immediate activation of NFκB and further induction of proinflammatory cytokines, TNFα and IL-6 (Adachi *et al.*, 1998). MyD88 recruits adaptor protein such as, TIRAP, TNF receptor-associated factor (TRAF) 6 and IL-1 receptor associated kinases (IRAKs), which have an essential role in the activation of NFκB and MAPK signaling (Kawagoe *et al.*, 2008; Kawai & Akira, 2010). In particular, IRAK4 is a critical adaptor

protein for MyD88-dependent signaling. For instance, macrophages deficient in IRAK4 demonstrate significant impairment and stability of cytokine production (Suzuki *et al.*, 2002; Kim *et al.*, 2007b; Lye *et al.*, 2008). Reports suggest that IRAK4 is responsible for recruiting other IRAK adaptor proteins to facilitate adequate proinflammatory cytokine expression (Lye *et al.*, 2004).

The TRIF-dependent pathway recruits adaptor proteins- TRAF3, TRAF6, and TRADD- that activate IFN regulatory factor-3 (IRF3), MAPKs and NFkB signaling (Pobezinskaya *et al.*, 2008). IRF3 signaling regulates IFN-β production and innate antiviral responses (e.g. viral replication), which is specific to TLR3 and TLR4 (Doyle *et al.*, 2002; Toshchakov *et al.*, 2002). TRIF-dependent pathway results in a delayed activation of NFκB and robust induction of IFN inducible genes, such as immunoresponsive gene 1 (IRG1) and a gene encoding CXCL10 (Kawai *et al.*, 1999; Kawai *et al.*, 2001).

Activation of MyD88 and TRIF-dependent pathways is counterbalanced by negative regulators of TLR signaling (Lu *et al.*, 2008). For instance, the toll-interacting protein (Tollip) is a critical negative regulator of TLR signaling. Tollip may bind with TLR2/TLR4 or IRAKs suppressing TLR-mediated immune responses (Zhang & Ghosh, 2002; Li *et al.*, 2004; Didierlaurent *et al.*, 2006). Single-Ig-interleukin 1 related receptor (SIGIRR) also a negative regulator by interacting with TLR4 to interfere with the recruitment of MyD88 (Wald *et al.*, 2003; Qin *et al.*, 2005a; Watson *et al.*, 2010). Taken together, whether TLR activation is MyD88- and/or TRIF-dependent, signaling solely depends on the availability of adaptor proteins and negative regulators in a given cell type.

L. Toll-like Receptor 4

Toll-like receptor 4 was the first characterized mammalian Toll (Medzhitov *et al.*, 1997). TLR4 is a highly studied PAMP receptor and serves to initiate a pro-inflammatory cascade in response to various exogenous and endogenous ligands. The most prominent exogenous ligand is LPS derived from gram-negative bacteria. A point mutation in the *tlr4 gene* was identified in C3h/HeJ mice that was hyporesponsive to LPS and further confirmed in TLR4-deficient mice (Poltorak *et al.*, 1998; Hoshino *et al.*, 1999). Endogenous ligands or DAMPs/ “alarmins” associate with TLR4; including fibrinogen, heparin sulfate, HSPs, and HMGB1 (Oppenheim & Yang, 2005; Bianchi, 2007; Yu *et al.*, 2010).

The leucine rich repeats (LRR) in the extracellular domain of TLR4 form a horseshoe structure with a hydrophobic core forming the ligand-binding site to the concave surface (Kim *et al.*, 2007a). TLR4 homodimerization may involve two accessary proteins, CD14 (glycosylphophatidylinositol-linked anchoring protein) and MD2 (myeloid differentiation protein-2; accessory secreted glycoprotein) (Wright *et al.*, 1990; Haziot *et al.*, 1996; Shimazu *et al.*, 1999). TLR4 can form a complex with MD2 on the plasma membrane that facilitates agonist binding(Park *et al.*, 2009; Hutchinson *et al.*, 2010b). CD14 has been shown to bind and deliver LPS to the TLR4 binding pocket, however, TLR4 can signal in the absence of CD14 primarily through MyD88-dependent pathway (Jiang *et al.*, 2005). In fact, TLR4 is unique for it can signal via both Myd88- and TRIF-dependent pathways **(Figure 5)** (Kawai *et al.*, 1999; Doyle *et al.*, 2002; Yamamoto *et al.*, 2002; Yamamoto *et al.*, 2003).

1. Subcellular localization and trafficking of TLR4

To date, TLR4 localization and trafficking mechanisms are primarily based on LPS-TLR4 signaling. In resting cells, TLR4-MD2-CD14 complex cycles between the plasma membrane and the Golgi until engaged at the surface by LPS (Thieblemont & Wright, 1999; Latz *et al.*, 2002). Upon activation at the plasma membrane, in the presence of TIRAP, MyD88 dependent signaling activates the NFκB pathway. In addition, TLR4 association with the adaptor protein- TRAM recruits TRIF to the plasma membrane resulting in translocation to endosome/lysosomes in a dynamin and clathrin-dependent manner (Husebye *et al.*, 2006; Kagan *et al.*, 2008; Tanimura *et al.*, 2008). TLR4/TRAM-TRIF dependent signaling is mediated in early endosomes activating the interferon regulatory factor (IRF) 3 pathway, which is similar to the nucleic acid sensing TLRs **(Figure 5)** (Kagan *et al.*, 2008). TLR4 and TRAM are further trafficked to late endosomes where TAG promotes TLR4 ubiquitination and Triad3A mediated degradation (Chuang & Ulevitch, 2004; Medvedev *et al.*, 2007; Palsson-McDermott *et al.*, 2009).

Figure 5:

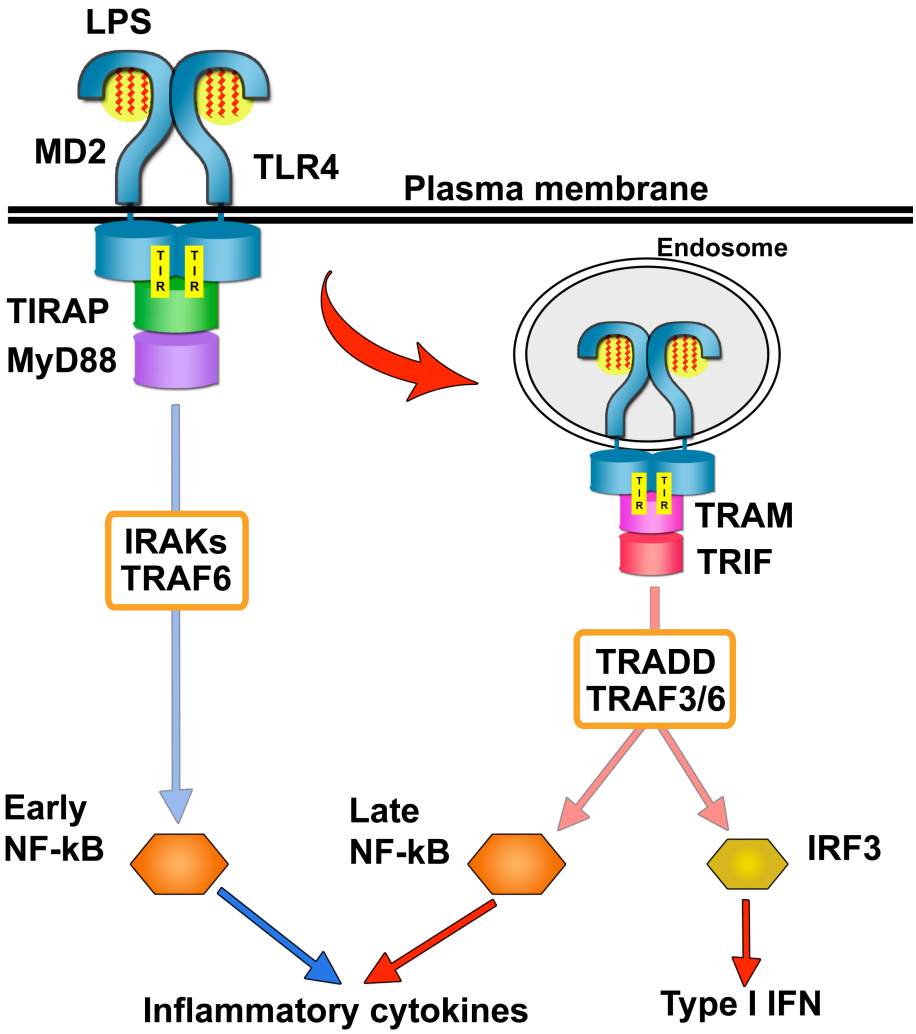


Figure 5: Toll like receptor 4 activation and signaling. Upon activation, LPS engages with TLR4 in complex with MD2. The receptor complex is composed of two copies of the TLR4-MD2-LPS that initially transmits signals for the early-phase activation of NFκB by recruiting the TIR domain containing adaptors TIRAP and MyD88 (MyD88-dependent pathway). The TLR4-MD2-LPS complex is then internalized and retained in the endosome, where it triggers signal transduction by recruiting TRAM and TRIF, which leads to the activation of IRF3 and late-phase NFκB for the induction of type I interferon (TRIF-dependent pathway). Both early- and late-phase activation of NFκB is required for the induction of inflammatory cytokines. Other adaptor proteins include: IRAKs, TRAF6, TRADD, and TRAF3/6.

2. Innate and adaptive immune system

The innate immune system works in conjunction with the adaptive immune system to maintain physiological homeostasis and protect the host against potentially pathogenic organisms. TLR4 as a PAMP, recognizes various invasive microorganisms as well an endogenous molecules that result in the activation of the innate and adaptive immune system (Medzhitov *et al.*, 1997; Poltorak *et al.*, 1998). Since, TLR4 is expressed on monocytes, macrophages, poly-morphonuclear cells, dendritic cells, this provides a great deal of flexibility for the immune system to respond accordingly (Hornung *et al.*, 2002; Zarember & Godowski, 2002; Caramalho *et al.*, 2003; Shimura *et al.*, 2005). TLR4 is largely dedicated to detecting bacterial infections; however, TLR4 can also detect viral infections, such as respiratory syncytial virus (RSV) and rhabdoviral (vesicular stomatitis virus; VSV) infections (Kurt-Jones *et al.*, 2000; Georgel *et al.*, 2007). Overall, immune cells that are activated by TLR4 contribute to infection, inflammation, wound healing, and tissue injury. TLR4 expression is found on both immune (microglia) and non-immune cell types (glial cells and neurons) that collectively influence immune surveillance, pro-inflammatory cytokine production, and neurodegeneration (Goethals *et al.*, 2010).

3. Central nervous system

Depending on the central nervous system (CNS) disease, TLR4 has been implicated in contributing to microglia activation, astrocyte dysfunction, neurotoxicity and neuronal excitability to influence a number of CNS pathologies;

such as, autoimmune encephalitis, stroke, Alzheimer's disease, Multiple Sclerosis, epilepsy, traumatic brain or spinal cord injury. TLR4 is expressed on astrocytes, microglia, neural progenitor cells, cortical, hippocampal, and motor neurons (Bowman *et al.*, 2003; Qin *et al.*, 2005b; Rolls *et al.*, 2007; Tang *et al.*, 2007; Shechter *et al.*, 2008)

Astrocytes form the blood-brain barrier, regulate cerebral blood flow, and are involved in the tripartite synapse. TLR4 on astrocytes are constitutively expressed and activation of astrocytic TLR4 induces pro-inflammatory mediators (Carpentier *et al.*, 2005; Gorina *et al.*, 2011). Originating from primitive myeloid precursors, microglia are the resident immune cells and comprise approximately 10% of the total cells within the CNS. Microglial TLR4 is important for generating an anti-bacterial response. *In vitro* exposure of LPS, TLR4 agonist, to microglial cultures produces a robust induction of pro-inflammatory cytokine, TNFα and is toxic to neurons and oligodendrocytes (Lehnardt *et al.*, 2002; Lehnardt *et al.*, 2003; Qin *et al.*, 2005b). Additionally, the release of endogenous TLR4 ligands, such as HSP60, from injured cells mediates neurodegeneration in the CNS by TLR4-dependent microglia activation (Lehnardt *et al.*, 2008).

Recent evidence suggests TLR4 activation can directly alter neuronal function in the CNS. The direct activation of TLR4 in adult cortical neurons induces heightened excitability in the form of seizure activity (Sayyah *et al.*, 2003; Galic *et al.*, 2008; Maroso *et al.*, 2010). Additionally, the activation of TLR4 by HMGB1 and LPS has been reported to attenuate LTP in hippocampal slices

(Costello *et al.*, 2011). Collectively, TLR4 activation in the CNS is cell-type specific and influences a number of neurophysiological mechanisms.

4. Peripheral nervous system

In the periphery, TLR4 is involved in many biological functions including peripheral nerve degeneration/regeneration, wound healing, and tissue inflammation/ damage. TLR4 is expressed in Schwann cells, myocytes, epithelial cells, keratinocytes, fibroblasts, and sensory neurons (Frantz *et al.*, 1999; Cario *et al.*, 2000; Tabeta *et al.*, 2000; Song *et al.*, 2002; Wadachi & Hargreaves, 2006; Lee *et al.*, 2007; Acosta & Davies, 2008). A break in the epithelial barrier allows pathogens to enter, which leads to TLR4 activation of PNS-resident macrophages and initiates the inflammatory response to clear the infection. During chronic inflammation, such as rheumatoid arthritis, chronic TLR4 activation of synovial fibroblasts may lead to progressive cartilage and bone destruction (Brentano *et al.*, 2009).

Furthermore after a nerve injury, initial clearance of myelin debris by infiltrating macrophages is critical for axonal degeneration. In TLR4-deficient mice, chemoattractant MCP1 and macrophage recruitment were diminished at the distal nerve stump following a nerve crush injury (Boivin *et al.*, 2007). In contrast, injection of TLR4 agonist hastened clearance of degenerating myelin by recruiting greater numbers of macrophages (Vallieres *et al.*, 2006). Taken together, at the site of injury TLR4 activation has a critical role in promoting

clearance of myelin debris by facilitating macrophage recruitment and thereby supporting nerve regeneration.

TLR4-dependent neuronal excitability is not limited to the CNS, as primary sensory neurons exposed to the TLR4 specific endotoxins (i.e. LPS or morphine-3-glucuronide (M3G)) produces varying degrees of increased excitability (Ochoa-Cortes *et al.*, 2010; Due *et al.*, 2012), concentration-dependent increase in intracellular calcium, inward currents, and the release of CGRP (Hou & Wang, 2001; Qin *et al.*, 2004).

5. TLR4 and pain

TLR4 responds rapidly to stressors induced by nerve injury to generate a host of proinflammatory mediators that can influence acute and chronic pain states. For instance, intrathecal administration of LPS generates acute mechanical hypersensitivity lasting up to an hour after injection (Hutchinson *et al.*, 2009; Saito *et al.*, 2010). Interestingly in an animal model of arthritis, TLR4 activation was shown to promote the transition from acute to chronic post-inflammatory pain hypersensitivity (Christianson *et al.*, 2011). Using the K/BxN serum transfer arthritis model, TLR4-deficient mice displayed a significant reversal in mechanical hypersensitivity after resolution of peripheral inflammation compared to wild-type mice (Choe *et al.*, 2003; Christianson *et al.*, 2011). Collectively, TLR4 activation may contribute to acute and chronic inflammatory pain states.

Moreover, the involvement of TLR4 in the generation of neuropathic pain is especially highlighted by observations of both diminished spinal cord inflammation and pain behavior hypersensitivity in TLR4 knockout mice following a peripheral nerve injury (Tanga *et al.*, 2004; Tanga *et al.*, 2005). TLR4 knockout mice fail to display behavioral hypersensitivity as early as one day after L5 spinal nerve ligation (Tanga *et al.*, 2004; Tanga *et al.*, 2005). Similar observations are evident in rats treated with TLR4 antisense oligodeoxynucleotides or siRNA following CCI (Tanga *et al.*, 2005; Wu *et al.*, 2010a).

A recent study by Sorge and colleagues discovered a pronounced gender difference in TLR4 activation and pain outcomes. Initial experiments determined that intrathecal injections of LPS may produce robust mechanical allodynia, but only in male mice. Interestingly, LPS administered through intracerebro-ventricular or intraplantar routes was able to produce equivalent allodynia in both sexes (Sorge *et al.*, 2011), suggesting TLR4-mediated inflammatory pain may have a gender specific bias in the spinal cord. Furthermore, after spared nerve injury it was shown that only male mice benefited from a TLR4/MD2 antagonist (Sorge *et al.*, 2011). With the exception of the findings by Sorge and colleagues, neuropathic pain models testing TLR4 agonists utilized male rodents and all displayed similar reversal of injury-induced hypersensitivity **(Table 4)**.

Thus, the type of injury may impact the role of TLR4 in neuropathic pain. Tanga and colleagues first described that following CCI TLR4-deficient mice displayed diminished pain hypersensitivity, which was attributed to a decrease in microglia activation (Tanga *et al.*, 2004; Tanga *et al.*, 2005). However, after a SNI

injury, TLR4-deficient male mice developed pronounced pain hypersensitivity, suggesting TLR4-independent or compensatory events by other TLRs (Sorge *et al.*, 2011). For instance, TLRs 3,7 and 9 are also expressed on human DRGs. Activation of TLRs 3, 7, and 9 have the ability to upregulate the expression of TRPV1, and enhance calcium flux by TRPV1-expressing DRGs (Qi *et al.*, 2011). Further, TLR3 antisense oligodeoxynucleotides are also capable of suppressing nerve injury-induced tactile allodynia (Obata *et al.*, 2008). Overall, additional research is needed to discern the differences in TLR4 activation after a chronic constriction injury, spared nerve injury and gender. **Table 4** provides a summary of relevant rodent pain behavior studies examining TLR4.

Table 4:

Injury	Animal	Route of Administration	Mechanical Allodynia	Thermal Hyperalgesia	Reference
Naïve	S/D M	i.t. LPS (10μg)	Pain 30-60 min after LPS injection	N/A	Saito, 2010
Naïve	S/D M	i.t. LPS (2μg, 100μg)	No effect long term >24hrs	N/A	Hutchinson, 2009
Naïve	CD1	i.t. LPS (0.1μg)	M 1-6hrs, F no effect	N/A	Sorge, 2011
Naïve	CD1	i.c.v. LPS (dose-response)	No effect M or F	N/A	Sorge, 2011
Naïve	CD1	i.pl. LPS (dose-response)	No effect M or F	N/A	Sorge, 2011
Naïve	CD1	i.t. LPS (0.1μg), LPS-RS (2μg) -6hrs post LPS injection	M 10-120min after LPS injection	N/A	Sorge, 2011
CFA	CD1, SCNJ (M-WT)	i.t. LPS-RS (2μg)	M reversal, F no effect	N/A	Sorge, 2011
CFA	TLR4 KO (M)	i.t. LPS-RS (2μg)	No effect	N/A	Sorge, 2011
CCI	S/D M	i.t. LPS-RS (2μg)	Diminshed 1, 3hr, PID 14	N/A	Hutchinson, 2008
CCI	S/D M	s.c.(+)- or (-)- naloxone (100mg/kg)	Reversed	N/A	Hutchinson, 2008
CCI	S/D M	s.c. HSP90 inhibitor geldanamycin (HSP90-TLR4 cofactor)	Diminshed pain, 3hr PID 10-14	N/A	Hutchinson, 2009
CCI	S/D M	i.t. 17-DMAG (HSP90 inhibitor)	Diminshed pain, 1hr PID 10-14	N/A	Hutchinson, 2009
CCI	S/D M	i.t.TLR4 siRNA, daily, start day 1 before surgery for 7 days	Diminshed pain, PID 1-7	Dimished pain, PID 1-7	Wu et al, 2010
CCI	S/D M	i.t. MD2-Inhbitor	Dimished pain 3hr both sides	N/A	Liu, 2011
CCI	S/D M	i.t. epigallocatechin gallate (TLR4 inhibitor)	Reversed, PID 1-14	Reversed, PID 1-14	Kuang, 2012
CCI	C57/BLK6 (M)	i.p. (7x one day post-CCI) FP-1 TLR4 antagonist (5, 10mg/kg)	Dimished pain, PID 4, 8	Dimished pain, PID 4, 8	Bettoni, 2008
CCI	TLR4 KO (M)	N/A	Dimished pain, PID 4, 8	Dimished pain, PID 4, 8	Bettoni, 2008

Table 4 continued:

Injury	Animal	Route of Administration	Mechanical Allodynia	Thermal Hyperalgesia	Reference
SNL	S/D M	s.c. (-)-naloxone (100mg/kg)	Reversed, PID 28	N/A	Lewis, 2012
SNL	S/D M	s.c. (+)-naloxone (100mg/kg)	Reversed, PID 14	N/A	Lewis, 2012
L5 SNT	SCNJ	N/A	Diminished pain, PID 1-14	Diminished pain, PID 1-14	Tanga, 2005
L5 SNT	C3H/HeJ	N/A	Diminished pain, PID 1-14	Diminished pain, PID 1-14	Tanga, 2005
L5 SNT	S/D M	i.t. TLR4 antisense oligo, daily, start day 1 before surgery for 7 days	Diminshed pain, PID 1-7	Diminshed pain, PID 1-7	Tanga, 2005
SNI	CD1, SCNJ (M-WT)	i.t. LPS-RS (2μg)	M reversed, F no effect	N/A	Sorge, 2011
SNI	TLR4 KO (M)	i.t. LPS-RS (2μg)	Pain behaivor before/after LPS-RS	N/A	Sorge, 2011
K/BxN arthritis	TLR4 KO (M)	N/A	Reversal after resolution of inflammation	N/A	Christianson, 2011
K/BxN arthritis	C57/BLK6 (M)	i.t. LPS-RS (2μg)	Diminished pain PID 6,8,12 after serum transfer	N/A	Christianson, 2011
Bone cancer pain	S/D M	i.t. (3x) TLR4 siRNA, behavior: 24hr after last injection	Diminished pain, PID12	N/A	Lan, 2010

Table 4: Rodent pain behavior studies examining TLR4.

M. Hypothesis and specific aims

Peripheral neuropathic pain is a debilitating disease that dramatically impacts quality of life. This type of pain is often due to post-operative complications, mechanical trauma, disease, and/or neurotoxic chemicals. After a precipitating event, such as nerve injury, pathophysiological changes in nociceptive primary sensory neurons are thought to initiate a cascade of events leading to a prolonged hyperexcitable state or neuronal hypersensitivity. A number of factors present in the peripheral environment may serve to perpetuate this peripheral sensitization and may include pro-nociceptive factors released from sensory neurons, glial cells or innate immune cells. On the other hand, there is the possibility of a signal that can be released by all cells associated with injury to the peripheral environment. A molecule that meets this criteria is HMGB1.

HMGB1 is an intracellular protein that is primarily localized to the nucleus of most cells and binds DNA to regulate gene expression; however, in times of cellular stress, HMGB1 is released as an inflammatory cytokine (Wang *et al.*, 1999). The cellular secretion of HMGB1 may serve to alert neighboring cells of tissue injury and initiate protective responses (Bianchi & Manfredi, 2004). To date HMGB1 signaling has been associated with diseases such as sepsis, cardiomyopathy, diabetes, and neuropathologies (Bierhaus *et al.*, 2004; Buchanan *et al.*, 2010; Huang *et al.*, 2010; Qiu *et al.*, 2010).

The effects of extracellular HMGB1 on cells are mediated by its two best-known receptors, RAGE and TLR4. HMGB1 acts on TLR4 to activate

proinflammatory signaling cascades in immune cells such as macrophages; however, HMGB1 activation through TLR4 is not limited to non-neuronal cells. Recent publications demonstrate that neurons, including pain transmitting primary sensory neurons, exhibit TLR4 (Chakravarty & Herkenham, 2005; Wadachi & Hargreaves, 2006; Acosta & Davies, 2008; Maroso *et al.*, 2010; Diogenes *et al.*, 2011). In the central nervous system elevated levels of HMGB1 in the brain induce memory abnormalities by either RAGE or TLR4 (Mazarati *et al.*, 2011), even acute application of HMGB1 has been shown to impair LTP (Costello *et al.*, 2011). Following injury or damage to the nervous system, HMGB1 interacting through TLR4 on cortical neurons can produce seizure activity (Maroso *et al.*, 2010); a hyperexcitable state not unlike the chronic spontaneous activity generated by primary afferent neurons that contribute to pain hypersensitivity in pain states. Collectively, excessive HMGB1 likely disrupts neurophysiological function that may contribute to nervous system disorders/diseases.

The ability of HMGB1 to influence neuropathic pain behavior was first demonstrated by Shibasaki and colleagues (Shibasaki *et al.*, 2010). They provided evidence that HMGB1 injected into the sciatic nerve not only produces hyperalgesic behavior, but intrathecal injection of a neutralizing anti-HMGB1 antibody, potentiated pain hypersensitivity after L5 SNL. Furthermore, TLR4 knockout mice and rats intrathecally injected with TLR4 antisense oligodeoxynucleotides fail to display behavioral hypersensitivity as early as one day after L5 SNL (Tanga *et al.*, 2004; Tanga *et al.*, 2005). Initial analysis of these

data suggested that the pain behavior attenuation was due to the absence of TLR4-dependent microglial activation in the spinal cord. However, given the observation that pain-sensing primary sensory neurons also exhibit TLR4 (Wadachi & Hargreaves, 2006; Acosta & Davies, 2008), it is plausible that chronic pain behavior following peripheral nerve injury is due to HMGB1 dependent neuronal TLR4 activation. Perhaps more importantly, HMGB1 may act as a physiological relevant source contributing to peripheral sensitization and neuronal hyperexcitability associated with neuropathic pain models.

The aim of this dissertation is to determine the degree to which HMGB1 serves to chronically sensitize nociceptive sensory neurons in the highly reproducible TNI model of peripheral neuropathic pain. The work explores the central hypothesis that peripheral sensitization of somatic sensory neurons in the rat following TNI is dependent on endogenous HMGB1 and TLR4. To investigate these goals, the following specific aims are proposed:

1. *Define TNI-induced changes in HMGB1 and TLR4 in primary afferent neurons*
2. *Determine the degree to which HMGB1 in vitro alters primary sensory neuron activity and function*
3. *Determine the degree to which HMGB1 and/or TLR4 is necessary for tactile hypersensitivity following TNI*

CHAPTER II

MATERIALS AND METHODS

The following section describes the methods and materials utilized in the experimental research sections (**Chapter III, IV**).

A. Animals

Pathogen-free, adult female Sprague Dawley (S/D) rats (150 to 200 g; Harlan Laboratories, Madison, WI, USA) were housed in temperature (23 ± 3°C) and light (12-hour light: 12-hour dark cycle; lights on at 07:00 hours) controlled rooms with standard rodent chow and autoclaved tap water available. Experiments were performed during the light cycle. Animals were randomly assigned to the treatment groups. All animal-related experiments were approved by the Institutional Animal Care and Use Committee of Indiana University School of Medicine. All procedures were conducted in accordance with the Guide for Care and Use of Laboratory Animals published by the NIH and the ethical guidelines of the International Association for the Study of Pain.

B. Rodent model of neuropathic pain: Tibial nerve injury

All rodents were anesthetized prior to surgical procedure with isoflurane (4% induction, 2% maintenance). To model neuropathic pain we performed a tibial nerve injury (Decosterd & Woolf, 2000; Lee *et al.*, 2000; Wang *et al.*, 2011b). S/D female rats 150 to 200g were anesthetized using isoflurane at 4%

induction and 2% maintenance. Under anesthesia, the right sciatic nerve was isolated under aseptic surgical conditions by blunt dissection of the femoral biceps muscle, without damaging the epimycium. The sciatic nerve and its three branches were isolated: the sural, common peroneal and tibial nerves. Only the tibial nerve was tightly ligated with 5-0 silk and transected distal to the ligation. An additional 2 to 4mm of distal nerve stump was removed to prevent re-innervation by the proximal nerve **(Figure 6)**. The overlying muscle and skin was then sutured in two separate layers. Sham-injured animals were subjected to all preceding procedures with the exception of ligation and transection.

Figure 6:

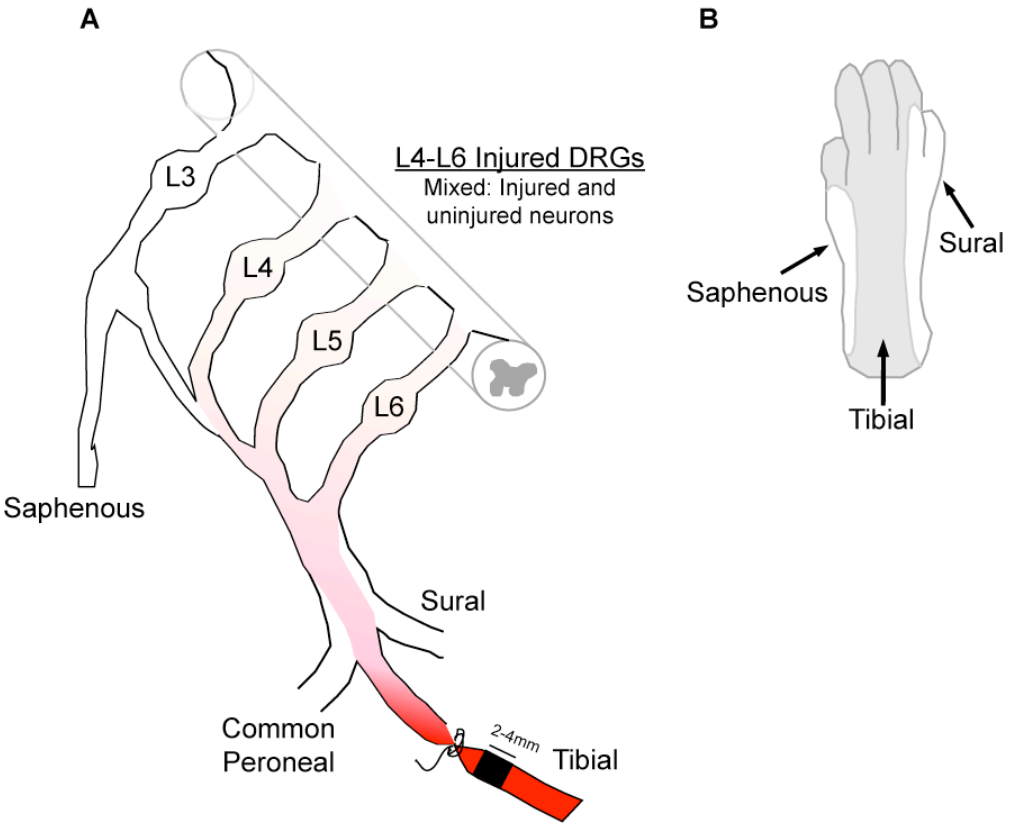


Figure 6: Schematic of tibial nerve injury. (A) The sciatic nerve is comprised of three branches: the sural, common peroneal and tibial nerve. Only the tibial nerve is transected and an additional 2-4mm of the distal nerve stump removed to prevent re-innervation. L4-L6 DRGs correspond to injured DRGs, which comprise both injured and uninjured sensory neurons. **(B)** Different zones of the plantar surface of the rat hindpaw innervated by the sciatic terminal branches. Sural (lateral), tibial (center), saphenous (medial). Saphenous nerve (femoral nerve plexus, L3 DRG) has minimal overlap with sciatic nerve branches (sciatic nerve plexus L4,5,6 DRGs). Adapted from (Decosterd, 2000).

C. Behavioral assessment of tibial nerve injury

The tibial nerve injury rodent model of neuropathic pain was assessed by thermal, cold, and tactile pain hypersensitivity. Tactile hypersensitivity after TNI produced reliable and robust pain hypersensitivity and was further assessed in experiments outlined in **Chapter III** and **IV**. All rodents were habituated to testing chambers for at least two days. Rodents were randomly assigned to sham or injured test group and baseline testing occurred before and after TNI. Upon completion of behavioral testing, animals were euthanized and tissue was collected for further analysis.

1. Thermal hyperalgesia

Thermal hyperalgesia was determined by measuring foot withdrawal latency and duration of paw withdrawal response to heat stimulation (Bhangoo *et al.*, 2007a). Each rat was placed in a box (22x12x12 cm) containing a smooth glass floor. A heat source (Ugo Basile Plantar™ Analgesia Instrument) was focused on the plantar surface of the hindpaw and a radiant thermal stimulus was delivered to that site. The stimulus shuts off automatically when movement of the hindpaw is detected or after 20s to prevent tissue damage. The intensity of the heat stimulus was constant throughout all experiments. A thermal stimulus was delivered 6 times to each hindpaw at five-minute intervals. The value for the response based on thermal latency and duration of paw withdrawal was obtained by averaging 5 of 6 measurements per animal.

Following TNI, the withdrawal response latency (in seconds) to nociceptive heat stimulation decreased at post injury day (PID) 11, 14, and 21 compared to baseline by 6.7s ± 0.22, 3.4s ± 0.31, and 4.0s ± 0.42 respectively in the hindpaw ipsilateral to the injury (**Figure 7A**; n=5, *P< 0.05). The duration of paw withdrawal in response to the heat stimulus increased significantly at PID 14 over baseline line levels for both the ipsilateral (injured) and contralateral (intact) hindpaw by 6.1s ± 2.2 and 4.8s ± 2.5, respectively (**Figure 7B**; n=5, *P< 0.05). Although a change in duration was evident at PID 14 in both hindpaws, where as a change in latency was evident at PID 11, 14, and 21 in the ipsilateral hindpaw, which may suggest that at PID 14 animals may experience heightened sensitivity or irritability to stimuli.

Figure 7:

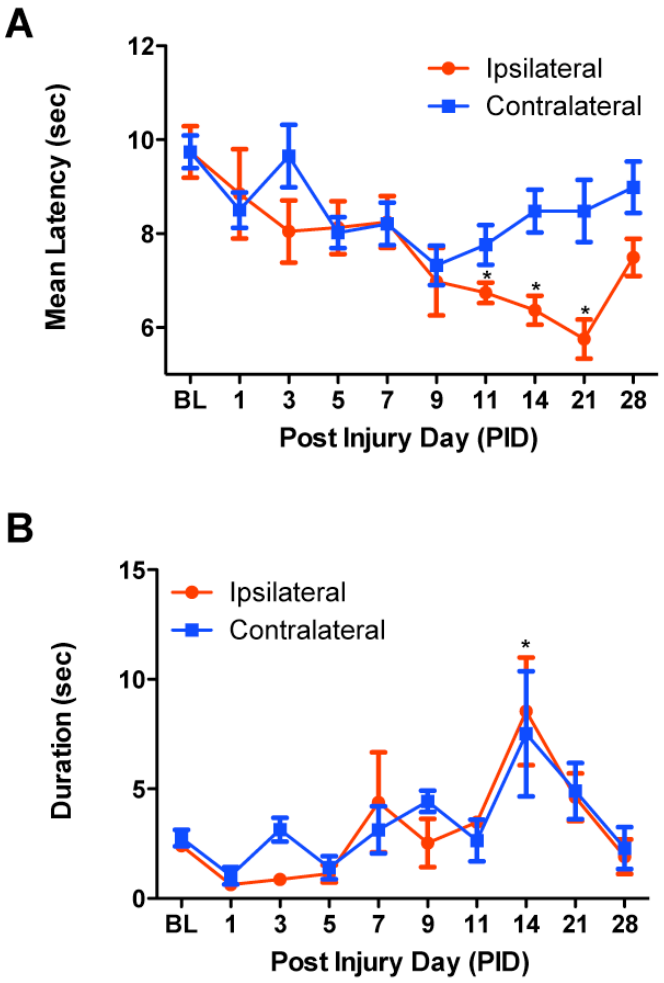


Figure 7: Behavioral assessment of thermal hyperalgesia following tibial nerve injury (TNI). (A) The paw withdrawal latency (in seconds) to nociceptive heat stimulation at PID 1-28 for the contralateral (intact) and ipsilateral (injured) hindpaw. TNI decreased the withdrawal response latency at PID 11,14, and 21 in the ipsilateral hindpaw. (n=5; ANOVA, F=4.47, Bonferroni multiple comparison test, *P< 0.05) **(B)** The duration of the withdrawal (in seconds) at PID 1-28 for the contralateral and ipsilateral hindpaw. TNI increased the duration of withdrawal at PID 14 for both hindpaws. (n=5; ANOVA, F=3.86-ipsilateral hindpaw, F=2.57-contralateral hindpaw, Bonferroni multiple comparison test, *P< 0.05). Data are expressed as mean ± s.e.m. *Significant differences compared with preoperative baseline control values (*P< 0.05). BL: baseline.

2. Cold allodynia

To quantify cold sensitivity of the hindpaw, rapid withdrawal in response to acetone applied five times (once every 5 minutes) to each paw was assessed (Lee *et al.*, 2000). A drop of acetone solution was carefully placed onto the plantar surface of the paw, using an angled syringe without touching the skin. The frequency of foot withdrawal expressed as a percentage was used as the cold allodynia index. The duration of withdrawal response was recorded with an arbitrary minimal value of 0.1s and a maximum of 30s (Choi *et al.*, 1994).

S/D female rats did not respond to acetone stimulation before tibial nerve surgery. After TNI, rats developed a marked hypersensitivity to acetone stimulation when applied to the hindpaw ipsilateral to the injury as early as one day after surgery and continued for each time point assessed PID 1-56. The hindpaw contralateral to the injury remained unresponsive to acetone (**Figure 8A**; n=3, **P*< 0.05). The duration of the withdrawal response significantly increased at later time points following injury, PID 14, 21, 28, and 42 in the hindpaw ipsilateral to the injury (13.4s ± 1.81, 23.18s ± 1.96, 16.91s ± 2.59, and 18.1s ± 2.37, respectively) compared to baseline (1.15s ± 0.40) (**Figure 8B**; n=3, **P*< 0.05). The duration of the paw withdrawal response in the hindpaw contralateral to injury was not affected by acetone stimulation.

Figure 8:

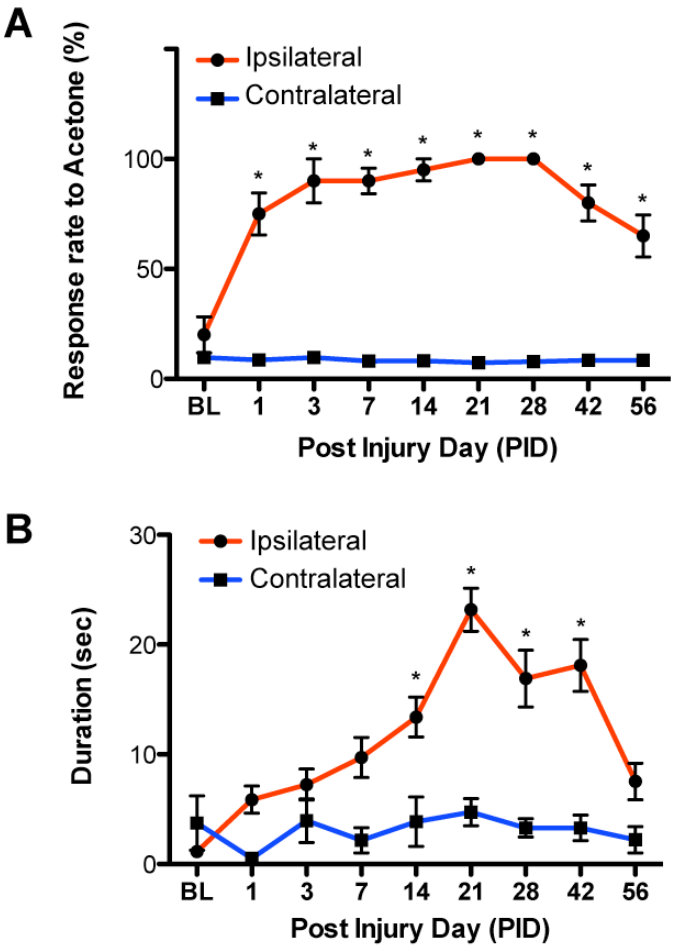


Figure 8: Behavioral assessment of cold allodynia following TNI. (A) Development of cold allodynia in rats following tibial nerve injury. Response rate to acetone was used as index of cold allodynia. Acetone stimulation elicited an increase in response rate at PID 1-56 (n=3; ANOVA, F=12.01, Bonferroni multiple comparison test, *P< 0.05). **(B)** The withdrawal duration (in seconds) after acetone stimulation to the hindpaw contralateral and ipsilateral to the nerve injury. The duration of the withdrawal response increased at PID 14, 21, 28 and 42. (n=3; ANOVA, F=14.99 Bonferroni multiple comparison test, *P<0.05). Data are expressed as mean ± s.e.m. Circles and squares indicated ipsilateral and contralateral sides, respectively. *Significant differences compared with preoperative baseline control values (*P< 0.05). BL: baseline.

3. Tactile hypersensitivity

The incidence of foot withdrawal in response to mechanical indentation of the plantar surface of each hindpaw was measured with a flat-tipped cylindrical probe or von Frey probe measuring 0.2 mm in diameter (Song *et al.*, 1999). Von Frey filaments capable of exerting forces of 10, 20, 40, 60, 80 and 120 mN with a uniform tip diameter was applied to a designated loci present on the plantar surface of the foot. During each test, the rodent was placed in a transparent plastic cage with a floor of wire with approximately $1cm^2$ openings. The cage was elevated so that stimulation was applied to each hindpaw from beneath the rodent. The filaments were applied in order of ascending force. For baseline measurements, von Frey probes were applied to 6 designated loci distributed over the plantar surface of the foot representative of sciatic nerve innervation into the hindpaw- saphenous, tibial, and sural (medial to lateral) (Song *et al.*, 1999; Ma *et al.*, 2003). Each filament was applied alternately to each foot for baseline measurements. Following TNI, von Frey probes were applied to 4 designated loci corresponding to most lateral regions of the hindpaw due to animal compensatory weight bearing on medial hindpaw (Wang *et al.*, 2011b). Each filament was applied ipsilateral to the nerve injury. The duration of each stimulus was approximately one second and the interstimulus interval was approximately 10 to 15 seconds.

The incidence of foot withdrawal was expressed as a percentage of the four applications of each stimulus and the percentage of withdrawals was then plotted as a function of force. A Hill equation (y=100*x^n/(k^n+x^n) was fitted

(Origin Version 6.0, Microcal Software) to the function relating the percentage of indentations eliciting a withdrawal to the force of indentation (Ma *et al.*, 2003). The von Frey withdrawal threshold (force corresponding to a 50% withdrawal) was defined as the force that evoked a minimum detectable withdrawal observed on 50% of the tests given at the same force level. For cases in which none of the specific filaments used evoked withdrawals on exactly 50% of the tests, linear interpolation will be used to define the threshold. Tactile hyperalgesia was defined as a postoperative decrease in threshold of 20 mN from the mean. TNI produces a robust increase in mechanical hypersensitivity that begins on day 3 and lasts for at least 4 weeks after surgery **(Figure 9)** (Wang *et al.*, 2011b).

Figure 9:

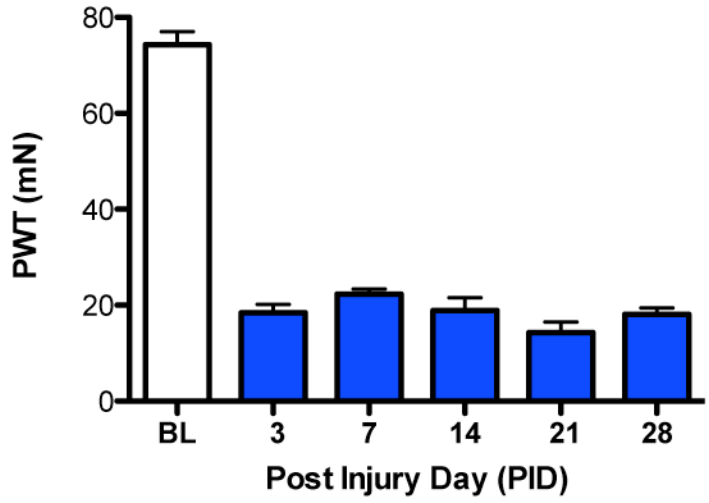


Figure 9: Behavioral assessment of tactile hypersensitivity following TNI. Tibial nerve injury results in a decrease in paw withdrawal threshold (PWT) beginning at day 3 after injury and each time point measured thereafter (PID 7, 24, 21, and 28) (n=6; ANOVA, F=125.2, Bonferroni multiple comparison test, *P< 0.05). Data are expressed as mean ± s.e.m. *Significant differences compared with preoperative baseline control values (*P< 0.05). BL: baseline.

D. Drug administration and treatment paradigm

Pre-TNI baseline behavioral assessment was established in all rodents. For some experiments, animals were injected with glycyrrhizin (GL; Sigma Aldrich, St. Louis, MO, USA). Glycyrrhizin is a natural anti-inflammatory and antiviral triterpene that binds directly to HMGB1 (Mollica *et al.*, 2007). Glycyrrhizin was prepared in saline solution on the day of the experiment (pH 7.5). Sham-control animals and TNI-induced animals were given intraperitoneal (i.p.) injections of saline (vehicle) or GL (50 mg/kg), respectively. Our dosing paradigm following TNI was either a single injection of GL or a once daily injection of GL for four consecutive days **(see Chapter III; Figure 17)**.

E. Tissue processing immunostaining

For immunohistochemistry, animals were sacrificed and transcardially perfused with saline followed by 4% paraformaldehyde. Fixed tissue was then embedded for sectioning and processed using immunocytochemical and immunohistochemical methodologies (Bhangoo *et al.*, 2007a). Lumbar L_4/L_5 dorsal root ganglia (DRG) tissue from naïve, sham control, and injured animals (ipsilateral to the nerve injury) were serially sectioned at 14μm for immunohistochemical experiments. At least 6 sections per DRG such that DRG sections on each slide were at intervals of 80μm.

F. Preparation of dissociated dorsal root ganglion neurons

The L_4 to L_6 DRGs, ipsilateral to the injury or sham-injury, were dissociated using methods described by Ma and LaMotte (Ma & LaMotte, 2005). Briefly, L_4 to L_6 DRGs were removed from sham or TNI animals at post-injury day (PID) 7, 14, and 28. The DRGs were treated with collagenase A and collagenase D Hanks' balanced salt solution (HBSS) for 20 minutes (1 mg/ml; Roche Applied Science, Indianapolis, IN, USA), followed by treatment with papain (30 U/ml; Worthington Biochemical Corp., Lakewood, NJ, USA) in HBSS containing 0.5 mM EDTA and cysteine at 35°C. The cells were then dissociated by mechanical trituration in culture media containing 1 mg/ml bovine serum albumin and trypsin inhibitor (Worthington Biochemical). The culture media was Ham's F-12 mixture, DMEM, supplemented with 10% fetal bovine serum, penicillin and streptomycin (100µg/ml and 100U/ml) and N2 (Life Technologies, Corp., Carlsbad, CA, USA). The cells were then plated on coverslips coated with poly-L lysine and laminin (BD Biosciences, Franklin Lakes, NJ, USA) and incubated for two to three hours before additional culture media was added to the wells. The cells were incubated for 12 to 15 hours to adhere at 37°C (with 5% CO_2). In some experiments primary sensory neuron cultures grown on coverslips and after experimental treatments were fixed with PBS/4% paraformaldehyde for 15 minutes.

G. F11 cell line

A F11 cell line was utilized to study a purely neuronal cell population. Typically, DRG population is comprised of a heterogeneous cell population of

neuronal and non-neuronal cells. The F11 cells represent a homogenous population, exhibiting markers and receptors of nociceptive sensory neuron lineage (Boland & Dingledine, 1990; Jahnel *et al.*, 2003) and exhibit neuronal activity in response to nociceptive stimuli (Bender *et al.*, 2005; Vetter & Lewis, 2010). F11 cells are a fusion product cell line of a mouse N18TG2 neuroblastoma and a rat DRG sensory neuron hybrid cell line (Francel *et al.*, 1987). F11 cells were grown on glass coverslips under 5% CO2 in Ham's F-12 medium supplemented with 20% fetal bovine serum (FBS; Hyclone Laboratories, Inc., Logan, UT, USA), 100 pM hypoxanthine/1 pM aminopterin/l2 pA4 thymidine, and 50 IU/ml of penicillin/streptomycin. Cells were differentiated preceding an experiment with Ham's F-12 medium supplemented with 50 ng/ml of NGF and 0.5 mM dibutyryl cyclic AMP (db-cAMP). F11 cells that were used for experiments were differentiated with NGF and db-cAMP for 48 hours prior to stimulation. In some experiments, F11 cells were cultured on coverslips and after experimental treatments were fixed with PBS/4% paraformaldehyde for 15 minutes.

H. Immunostaining

Fixed tissue sections, F11 cells or primary sensory neuron cultures were blocked with natural horse serum blocking buffer (Thermo Scientific SuperBlock® Blocking Buffer in PBS, natural horse serum (3% v/v), and Triton-X (0.4% (v/v)). Primary antisera used was the rabbit anti-HMGB1 (1:1,000; Sigma Aldrich), goat anti-TLR4 (1:1,000; sc-16240; Santa Cruz Biotechnology), rabbit anti-ATF3

(1:1,000; Santa Cruz Biotechnology), monoclonal anti-NeuN, polyclonal anti-CGRP, *Griffoniasimplicifolia* IsolectinB4 conjugated to FITC IB_4-FITC (1:1,000; Sigma Aldrich), and Hoescht nuclear stain (1:1,000; Invitrogen Corporation). Sections were incubated in secondary antibodies conjugated to CY3 or FITC (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Slides or coverslips were washed in PBS for 10 min (2X) before and after each incubation step and then coverslipped with a PBS/glycerol solution. The working concentration for each antibody was determined by performing a dilution series that displayed minimal background and autofluorescence. For each experiment, control sections were incubated with only primary or secondary antibody to ensure specificity of immunoreactivity.

I. Western blot analysis

Animals were sacrificed and transcardially perfused with saline and tissue was removed and frozen immediately with liquid nitrogen and stored at -80°C. The fresh frozen L_4/L_5 DRG tissue samples, ipsilateral to the injury, were homogenized in radioimmunoprecipitation assay (RIPA) buffer with protease/phosphatase inhibitors and protein concentration was determined using the bicinchoninic acid BCA protein assay (Thermo Fisher Scientific, Rockford, IL). Samples (40µg/lane) were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane. After incubation in 10% non-fat milk blocking solution overnight at 4°C, the membrane was probed with rabbit anti-HMGB1 (1:1,000;Sigma Aldrich), goat anti-TLR4 (1:1,000; sc-12511; Santa Cruz

Biotechnology) followed by incubation with horseradish peroxidase-coupled anti-rabbit secondary antibody (Jackson ImmunoResearch). The membrane was reprobed with a monoclonal anti-β actin antibody (1:5,000; Sigma-Aldrich, St. Louis, MO) or monoclonal antibody to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:5,000; Ambion Applied Biosystems). Immuno-positive bands were detected by enhanced chemiluminescence (ECL) and measured by a densometric analysis (Unscanit; Silk Scientific Inc., Orem, UT).

J. Nuclear and cytoplasmic extraction

Nuclear and cytoplasmic extracts were prepared using NE-PER Nuclear and Cytoplasmic Kits (Thermo Fisher Scientific). Fresh $L_{4/5}$ DRG tissue ipsilateral to the injury were collected and stored at 80°C. Monoclonal lamin B, nuclear protein, (1:1,000; Santa Cruz Biotechnology) and monoclonal αTubulin, cytoplasmic protein (1:1,000; Santa Cruz Biotechnology) were used as loading controls.

K. Extracellular HMGB1 release measurement

F11 neuronal cell line was differentiated for 48 hours in a 24 well plate. F11 neuronal cells were washed twice with a balanced sterile solution (BSS) [NaCl (140 mM), Hepes (10 mM), CaCl2 (2 mM), MgCl2 (1 mM), glucose (10 mM), KCl (5 mM)]. To stimulate F11 cells, a high concentration of potassium solution (50 mmol/L KCL, denoted as 50K hereafter) was prepared by adjusting concentration of KCl from 5 to 50, and NaCl from 145 to 100. 50K, balanced

saline solution (BSS), and ionomycin (2μM) were applied for one hour. Extracellular supernatants were collected and briefly spun. Samples were concentrated using a centrifugal filter device (Amicon Ultra 4-10K; Millipore Corp., Billerica, MA, USA). Western blot analysis was performed to detect HMGB1 protein levels in extracellular supernatants. Ponceau staining was used to assess efficiency of transfer of proteins to the nitrocellulose membrane prior to immunoblotting **(Figure 10)**. Additionally, ponceau staining was also used as to check equal loading as an alternative to standard intracellular housekeeping proteins, actin and GAPDH (Romero-Calvo *et al.*, 2010).

Figure 10:

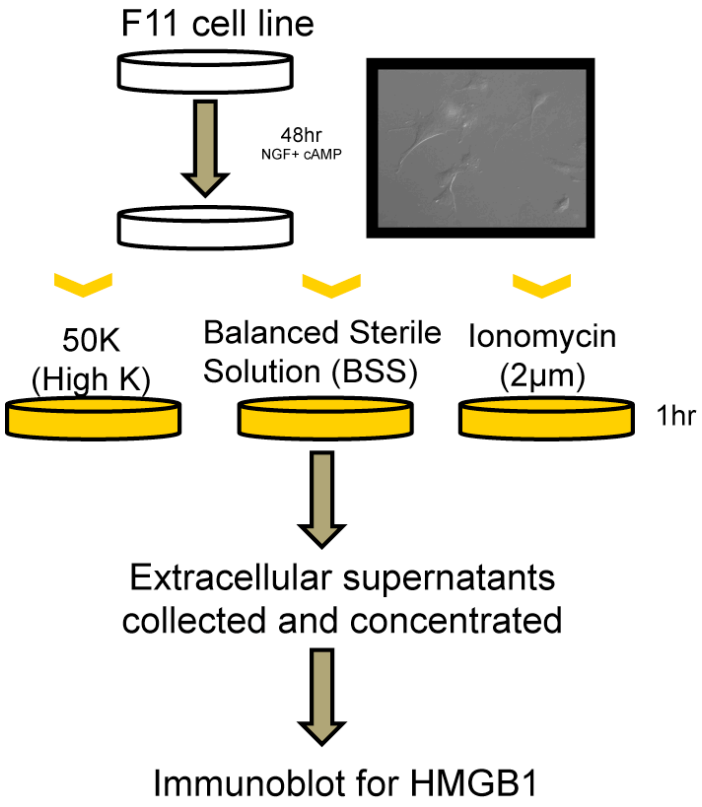


Figure 10: Diagram of assay for extracellular HMGB1 release measurement. F11 neuronal cell line was differentiated for 48 hours in a 24 well plate. F11 cells were stimulated with high concentration of potassium solution (50K), balanced saline solution (BSS) -as a negative control, and ionomycin (2µM) -as a positive control was applied for one hour. Extracellular supernatants were collected and briefly spun. Samples were concentrated followed by western blot analysis to detect HMGB1 protein levels in extracellular supernatants.

L. Cell count analysis

Images were taken with an intensified CCD camera (Photometrics CoolSnap HQ2) coupled to a Nikon microscope (Nikon Eclipse Ti) using Nikon Elements software (Nikon Instruments Inc., Melville, NY, USA). Tissue sections were illuminated with a Lamda DG-4 175 W xenon lamp (Sutter Instruments, Novata, CA, USA). The image of each section was set to a maximum threshold between 8000 and 8500, and total cell counts for each section were then taken using the grid function to aid in total cell count analysis. HMGB1, TLR4 and ATF3 immunopositive cell counts were conducted using Image Pro Software (Media Cybernetics, Inc., Bethesda, MD, USA). The following parameters were used for cell counts: intensity range (40 to 255), smoothness (20), and measurement window size (10μM). Fluorescent artifacts such as axons and cell debris were not included in cell counts. HMGB1, TLR4 and ATF3 immunopositive cell counts were taken from independent tissue section images and combined to reach the total percentage of neurons per ganglia. The criteria for neuronal HMGB1 cytoplasmic localization counts include: 1) presence of Hoescht nuclear label, and 2) complete cellular membrane morphology, and size of cell (> 10μm).

M. Cell size distribution

Cell size was measured using Nikon Elements Software (Nikon Instruments Inc., Melville, NY), long and short diameters (LD, SD) of each counted cell were measured and used to estimate cell cross-sectional area (area = p[LD/2] [SD/2], measured in lm^2). LD and SD were perpendicular to each other

and passed through the center of the cell soma. Neurons that were > 1000μm^2 in cross-sectional area were classified as large-sized neurons. They are presumed to maintain myelinated axons and mostly to function as low-threshold mechanoreceptors (Djouhri, Lawson 2004). Neurons < 1000μm^2 were classified as small and medium-sized cells (Nitzan-Luques *et al.*, 2011).

O. Recombinant HMGB1

Recombinant HMGB1 was purchased from R&D Systems (Minneapolis, MN; <1.0 endotoxin per 1g of the protein by the LAL method; 50% binding of biotinlyated HMGB1 at 0.35 to1.4μg/ml) and supplied as a 0.2μm filtered solution in PBS, EDTA and DTT (reducing agent). Stock solutions of HMGB1 were reconstituted in sterile PBS.

P. Intracellular calcium imaging

The dissociated DRG cells were loaded with fura-2AM (3mM, Invitrogen Corp., Carlsbad, CA USA) for 25 minutes at room temperature in a balanced sterile solution (BSS) (NaCl (140mM), Hepes (10mM), $CaCl_2$ (2mM), $MgCl_2$ (1mM), glucose (10mM), KCl (5mM). The cells were rinsed with the BSS and mounted onto a chamber that was placed onto the inverted microscope. Intracellular calcium was measured by digital video microfluorometry with an intensified CCD camera coupled to a microscope and MetaFluor software (Molecular Devices Corp., Downington, PA USA). Cells were illuminated with a 150W xenon arc lamp, and the excitation wavelengths of the fura-2AM (340/380

nm) were selected by a filter changer. Sterile solution was applied to cells prior to HMGB1 application; any cells that responded to buffer alone were not used in neuronal responsive counts. HMGB1 was applied directly into the coverslip bathing solution with a final concentration of 0.65µg/ml. After HMGB1 application, high potassium-50K (50mM) and capsaicin (3nM) were applied. Calcium imaging traces were analyzed by two independent analyzers and only responses that were in agreement between two individuals were used in the counts. Only calcium imaging traces that reflected at least a 50% increase over baseline were included in the analysis. A 50% increase over baseline was an arbitrary criteria set to reflect a response and minimize subjective variability in calcium image analysis. If no response or increased from baseline was seen within one minute, HMGB1 was washed out with BSS.

Q. Statistics

GraphPad Software (LaJolla, CA, USA) was used to determine the statistical significance. Results were expressed as mean ± s.e.m. When two groups were compared a Student's t-test was used. Multiple comparisons were evaluated by post-hoc Bonferroni test after one-way ANOVA. F values reflect a ratio of explained variance to unexplained variance. The F value is greater when the null hypothesis tested in not true. *P< 0.05 was considered to be statistically significant. GraphPad Software (LaJolla, CA) was used to determine the statistical significance of differences in calcium response among naïve and

injured groups using Chi-square test- yates correction, with a *P< 0.01 set as statistical significance.

CHAPTER III

THE PERSISTENT RELEASE OF HMGB1 CONTRIBUTES TO TACTILE HYPERALGESIA IN A RODENT MODEL OF NEUROPATHIC PAIN

A. Introduction

High mobility group box 1 protein is an 'alarmin' or DAMP molecule that rapidly mobilizes and activates innate and adaptive host immune defense mechanisms (Yang *et al.*, 2007). HMGB1 is a highly conserved 215 amino-acid non-histone nucleosomal regulatory protein that is important for DNA repair and replication. Though HMGB1 is typically associated with chromatin, it can be quickly released into the cytoplasm following injury. More importantly, the cytoplasmic HMGB1 can also act as a cytokine when released by macrophages following injury, inflammation, or disease (Wang *et al.*, 1999; Gardella *et al.*, 2002b; Vezzoli *et al.*, 2010). Recent studies have demonstrated that HMGB1 release is not limited to leukocytes but can also be released from activated or injured neurons (Vezzani *et al.*, 2011).

Release of HMGB1 by neurons in the CNS plays a crucial role as potential source of an endogenous inflammatory mediator that can influence adjacent neurons and glia (Kim *et al.*, 2006). Recent evidence also suggests that HMGB1 signaling in cortical cells may contribute to lower membrane thresholds and mediate rapid changes in neuronal excitability (Maroso *et al.*, 2010; Vezzani *et*

al., 2011). There is also the suggestion that HMGB1 may contribute to the development of neuropathic pain states (Shibasaki *et al.*, 2010). For example, perisciatic or intrathecal administration of HMGB1 produces rapid thermal hyperalgesia and mechanical allodynia in the rat (O'Connor *et al.*, 2003; Shibasaki *et al.*, 2010). In contrast, spinal nerve ligation-induced mechanical allodynia, but not thermal hyperalgesia, can be partially reversed if animals are pre-treated with intrathecal anti-HMGB1 antibody (Shibasaki *et al.*, 2010). Though HMGB1 may contribute to the development of neuropathic pain, the cellular source of HMGB1 that contributes to ongoing chronic pain behavior and the underlying role of HMGB1 in neuropathic pain are unknown.

In the present investigation, I examined the degree to which HMGB1 contributes to the peripheral sensitization of sensory neurons in a rodent TNI model of neuropathic pain. I observed that HMGB1 protein is upregulated in a number of sensory neurons for an extended period of time. Though not vesicle bound, release of HMGB1 from a neuronal cell line was found to occur in response to activity. Exogenous administration of HMGB1 increased excitability in acutely dissociated sensory neurons. Finally, I asked whether treatment with glycyrrhizin (GL) a natural anti-inflammatory and antiviral triterpene that binds directly to HMGB1 (Mollica *et al.*, 2007), could influence neuropathic pain behavior in the rodent. We found that GL effectively reverses TNI-induced mechanical allodynia both at fourteen days and three months following nerve injury.

B. Original experimental results

1. TNI induces cytoplasmic HMGB1 in many sensory neurons

HMGB1 is limited largely to the nuclei of non-neuronal cells and sensory neurons in the naïve DRG **(Figure 11A,A1)**. Following TNI, HMGB1 is observed in both the cytoplasm and nucleus of numerous sensory neurons in the L_5 DRG in addition to some non-neuronal cells at post injury day (PID) 14 **(Figure 11B,B1)**. The number of primary afferent neurons that exhibit cytoplasmic HMGB1 increased significantly when compared to naïve and sham-injured animals. The percentage of positive cytoplasmic HMGB1-immunoreactivity (IR) sensory neurons is increased after TNI at PID 14 compared to sham and naïve **(Figure 11C)**.

The expression of activating transcription factor 3 (ATF3), a cellular marker of nerve injury was used to reveal the primary afferent fibers that are engaged by TNI (Tsujino *et al.*, 2000; Braz & Basbaum, 2010). After TNI injury PID 14, ATF3-IR is present in 31.3% (126/403) of total L_5 DRG neurons ipsilateral to the injury (n=3) **(Figure 12B)**. ATF3 expression was present in heterogeneous population of sensory neurons. Sham and naïve DRGs did not exhibit ATF3 immunoreactivity **(Figure 12A)**. The relatively low number of ATF3 immunopositive sensory neurons in the sensory ganglia relative to the percentage of neurons exhibiting cytoplasmic HMGB1 immunoreactivity following TNI, suggests that direct nerve injury is not necessary for neuronal translocation of HMGB1.

Figure 11:

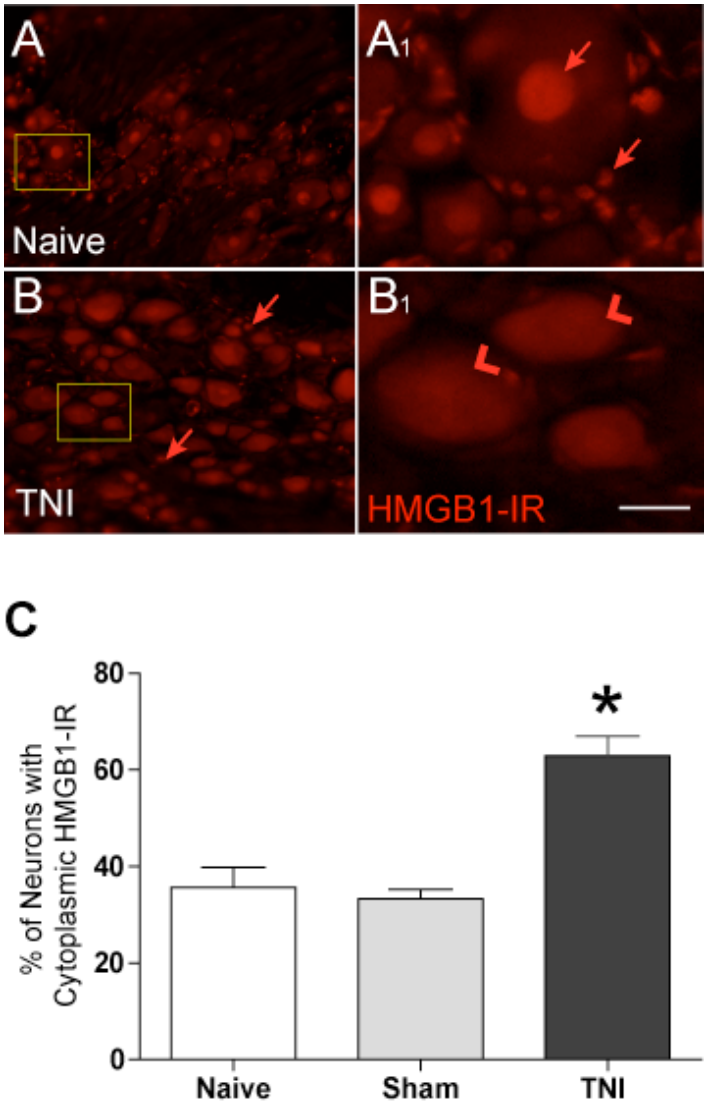


Figure 11: Subcellular localization of HMGB1 in L5 dorsal root ganglion (L_5 DRG) primary afferent neurons following TNI in rats. (A) Sections of L_5 DRG stained for HMGB1 immunoreactivity (IR) are localized primarily to the nuclei of both neuronal and non-neuronal cells (A1; arrows) in the naive L_5 DRG. Scale bar, 50µm. **(B)** By post-injury day (PID) 14, HMGB1-IR is localized to the cytoplasm of L_5 DRG neurons (B1; arrowheads). There are some nuclei of non-neuronal cells that also exhibit HMGB1-IR (B; arrows). **(C)** Cell counts performed on sections of sensory ganglia derived from naïve, sham and TNI animals revealed that a large number of sensory neurons exhibit HMGB1 in the cytoplasm (n=3 for each condition; ANOVA; Bonferroni's multiple comparison test, $*P< 0.01$.

Figure 12:

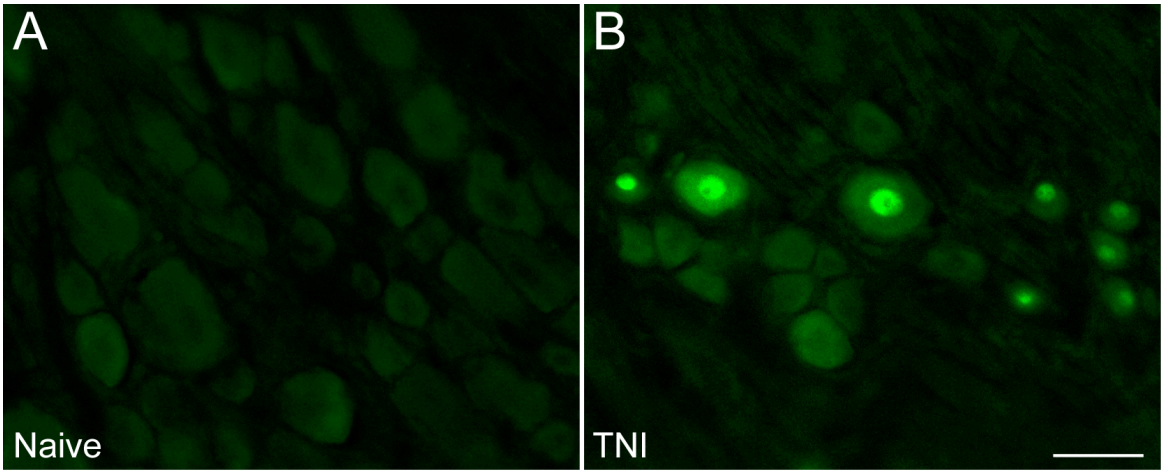


Figure 12: TNI-induced ATF3 expression in L_5 DRG primary afferent neurons. (A) Naïve sections of L_5 DRG stained for activating transcription factor 3 (ATF3)-IR. **(B)** By PID 14, ATF3-IR is localized to the nucleus of L_5 DRG neurons, n=3. Scale bar, 50μm.

To confirm that HMGB1 indeed exhibits a subcellular redistribution, nuclear and cytoplasmic extracts from $L_{4/5}$ DRGs ipsilateral to the injury were prepared from sham-injured and TNI animals at PID 14. Immunoblots of HMGB1 in the specific extracts revealed that there was a decrease in nuclear HMGB1 protein expression compared to sham control **(Figure 13B)** and an increase in cytoplasmic HMGB1 protein expression compared to sham control **(Figure 13A).** However, total HMGB1 protein content in the $L_{4/5}$ DRG was not altered by nerve injury **(Figure 14)**.

Figure 13:

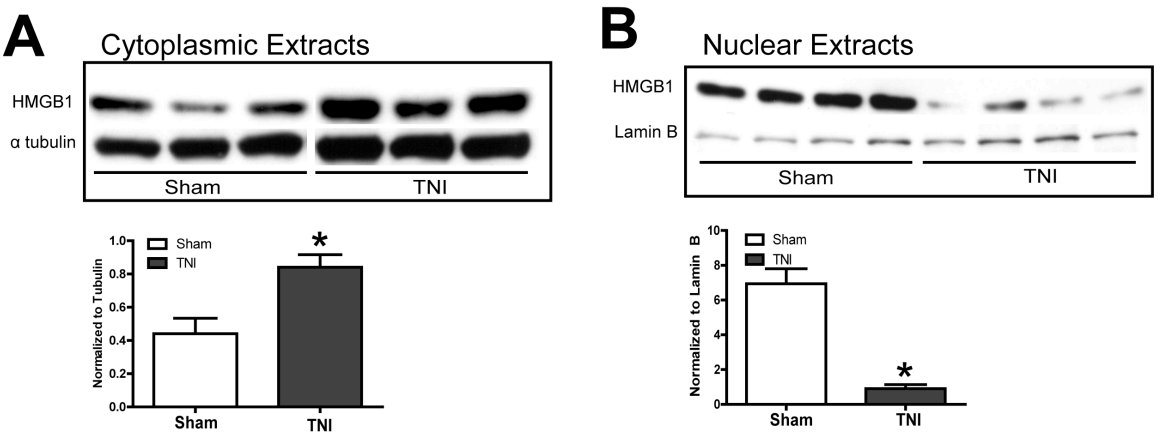


Figure 13: Distribution of HMGB1 in dorsal root ganglion tissue derived from TNI rats. Nuclear and cytoplasmic cellular compartments were extracted from L_4/L_5 DRGs ipsilateral to the injury. **(A)** HMGB1 levels in the cytoplasm increased compared to sham $L_{4/5}$ DRGs lysates post injury day (PID) 14 (n=3, Student's t-test, **P*< 0.05) **(B)** On the other hand, HMGB1 levels in the nucleus decreased compared to sham $L_{4/5}$ DRGs lysates PID 14 (n=4, Student's t-test, **P*< 0.01).

Figure 14:

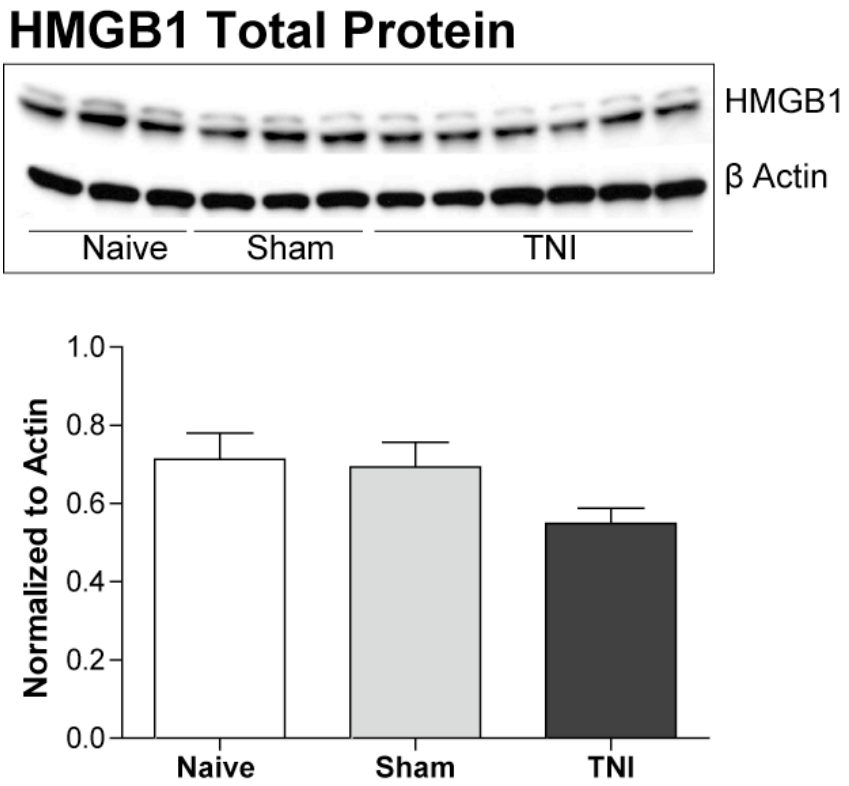


Figure 14: Total HMGB1 protein content in dorsal root ganglion tissue derived from TNI rats. Western blot analysis of total HMGB1 protein contents in naïve, sham-injured and TNI $L_{4/5}$ DRGs ipsilateral to the injury at PID 14. (naïve and sham n=3 each; TNI (PID 14) n=6, ANOVA, F=3.38; *P*> 0.05).

2. HMGB1 release in F11 cells is activity dependent

I used the F11 cell line as a surrogate sensory neuron to determine the ability of HMGB1 to function as a cytokine by testing whether HMGB1 is capable of extracellular release following exposure to high K+ -50 mM (50K). Not unlike naïve DRG sensory neurons, HMGB1-IR was present in the nucleus and absent in the cytoplasm of differentiated F11 cells **(Figure 15A,A_1)**. Following depolarization with 50K for one hour, HMGB1 accumulation was observed in the cytoplasm of numerous F11 cells **(Figure 15B,B_1)**. Moreover, HMGB1 was further released as evidenced by pronounced levels of HMGB1 in the extracellular supernatant **(Figure 16)**. Blebbing of F11 cell nuclei, an indicator of cell death, was absent following one-hour exposure to 50K. Despite evidence demonstrating extracellular release of HMGB1 from F11 cells, the combination of 50K and glycyrrhizin (GL; 100μM), a triterpenoid saponin glycoside known to neutralize HMGB1, did not affect HMGB1 nuclear translocation. Taken together, this suggests that the presence of HMGB1 alone may be sufficient but not necessary to mediate extracellular release upon neuronal stimulation.

Figure 15:

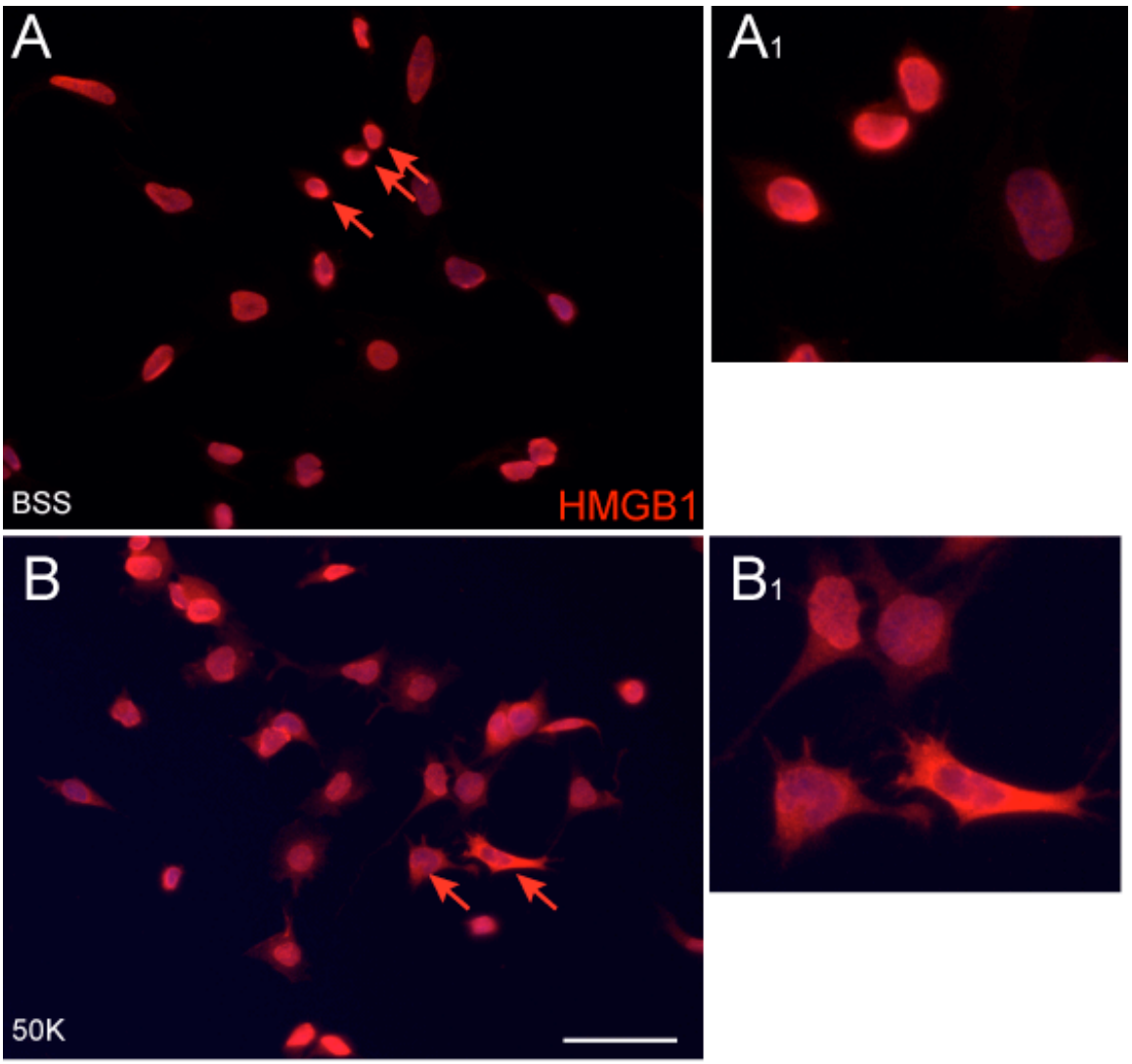


Figure 15: Immunofluorescent imaging of cytoplasmic HMGB1 from F11 neuronal cell line. (A, A_1) HMGB1 is confined in the nucleus of F11 differentiated neuronal cell line in the BSS control sample (arrows). **(B, B_1)** HMGB1 protein relocates into the cytoplasm following a one-hour exposure to 50K (arrows). Co-labeled with Hoechst stain-nuclei marker. Scale bar, 50μm.

Figure 16:

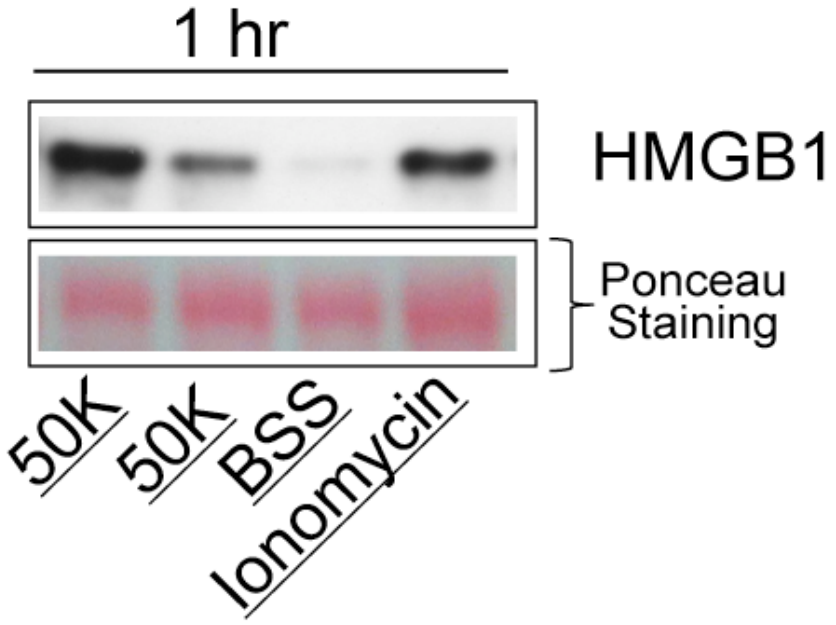


Figure 16: Extracellular release of HMGB1 from F11 neuronal cell line. Western blot analysis of extracellular supernatant of HMGB1 after a one-hour exposure to 50K, ionomycin (2μM) and a balanced sterile solution (BSS) of F11 differentiated cells. Extracellular supernatants exhibited an increase level of HMGB1 after 50K stimulation. Two sets of 12-well plates of cultured F11 cells were used. F11 cells exposed to 50K lane were duplicated within each 12-well plate. Lysates per condition and 12-well plate were loaded on a nitrocellulose membrane for HMGB1 detection by westernblot analysis. Nitrocellulose membrane was stained with ponceau staining, as an alternative to actin blotting. The 30kDa region is shown, which corresponds to molecular weight of HMGB1.

3. HMGB1 activates calcium mobilization in sensory neurons

Using calcium mobilization techniques **(see Chapter II)**, it is possible to functionally characterize sensory neurons that respond to acute administration of HMGB1. Following HMGB1 application, capsaicin (transient receptor potential cation channel subfamily V member 1;TRPV1 agonist) and high K+ (50K) (activates voltage-gated calcium channels) were added to further characterize the phenotype of the imaged cells. A response to 50K is indicative of a non-nociceptive neuron, while a response to capsaicin and 50K is characteristic of a nociceptive neuron. Numerous nociceptive neurons responded to HMGB1 while significantly fewer non-capsaicin neurons exhibited HMGB1-induced calcium mobilization **(Table 5)**.

As an additional control experiment, we addressed the feasibility of GL to effectively neutralize the direct effects of HMGB1 on sensory neurons. Using the described calcium mobilization paradigm, we bath applied acutely dissociated sensory neurons with GL/HMGB1. Following washout of GL/HMGB1, the cells were then exposed to capsaicin. The experimental outcome of these experiments suggested that most capsaicin-sensitive sensory neurons (>90%) did not respond to HMGB1 in the presence of 200 or 400μM GL.

Table 5:

	Naïve	
	Capsaicin-sensitive neurons	Non capsaicin-sensitive neurons
HMGB1	70% (31/44)	19% (26/136)

Table 5: Acute administration of HMGB1 elicits an intracellular calcium flux in primary sensory neurons. Dissociated naïve sensory neurons were incubated with fura-2AM to visualize calcium mobilization. HMGB1 was applied and response was measured in capsaicin and non-capsaicin sensory neurons. Capsaicin-sensitive neurons had a greater sensitivity to exogenous HMGB1. Excitation wavelengths of 340/380 nm were measured. Only calcium imaging traces that reflected at least a 50% increase over baseline were included in the analysis. Dissociated DRGs were incubated overnight in media that did not contain NGF. DRGs were isolated and calcium response was tested from 6 individual S/D rats (n=6).

4. HMGB1 increases the excitability of primary afferent neurons

Increased excitability of peripheral sensory neurons is thought to contribute to chronic pain states following nerve injury. To determine the degree to which HMGB1 can induce an increase in sensory neuron excitability, electrophysiology was performed using sharp electrodes in current clamp mode. All recordings were performed by Dr. Michael Due, for figures refer to (Feldman *et al.*, 2012). Following repeated current pulse combined with HMGB1 application, a significant increase in the excitability of some small to medium diameter sensory neurons was observed compared to baseline levels in both naïve (14.3% cells respond to HMGB1; 1.36 action potentials (APs) for control vs. 5.22 APs for HMGB1; (Feldman *et al.*, 2012)) and TNI derived sensory neurons (37.5% cells respond; 1.20 APs for control vs. 7.33 APs for HMGB1 (Feldman *et al.*, 2012)).

5. Glycyrrhizin reduces pain hypersensitivity in the TNI model of neuropathic pain

TNI produces a significant reduction in the paw withdrawal threshold (PWT) to tactile stimulus which lasts for several months (PID 3 to 64) (Wang *et al.*, 2011b). To investigate the degree to which HMGB1 modulates TNI-induced tactile hyperalgesia, we utilized a treatment paradigm described by Ohnishi and colleagues (Ohnishi *et al.*, 2011) that included either a one-time injection of glycyrrhizin (GL) via an intraperitoneal (i.p.) route, (50 mg/kg) at PID 7 or one injection per day over four consecutive days, PID 11 to 14 **(Figure 17A)**. This same treatment paradigm was again repeated over four consecutive days, PID 61 to 64 **(Figure 17B)**. Interestingly, a single injection of glycyrrhizin produced only a partial reduction in the PWT to tactile stimulus at PID 7 and PID 56 **(Figure 17C,D)**. However, four consecutive days of glycyrrhizin at either PID 11 to 14 or PID 61 to 64, produced PWTs that returned nearly to pre-injury baseline levels. This behavior represented strongly significant differences when compared to vehicle controls **(Figure 17C,D)**.

Figure 17:

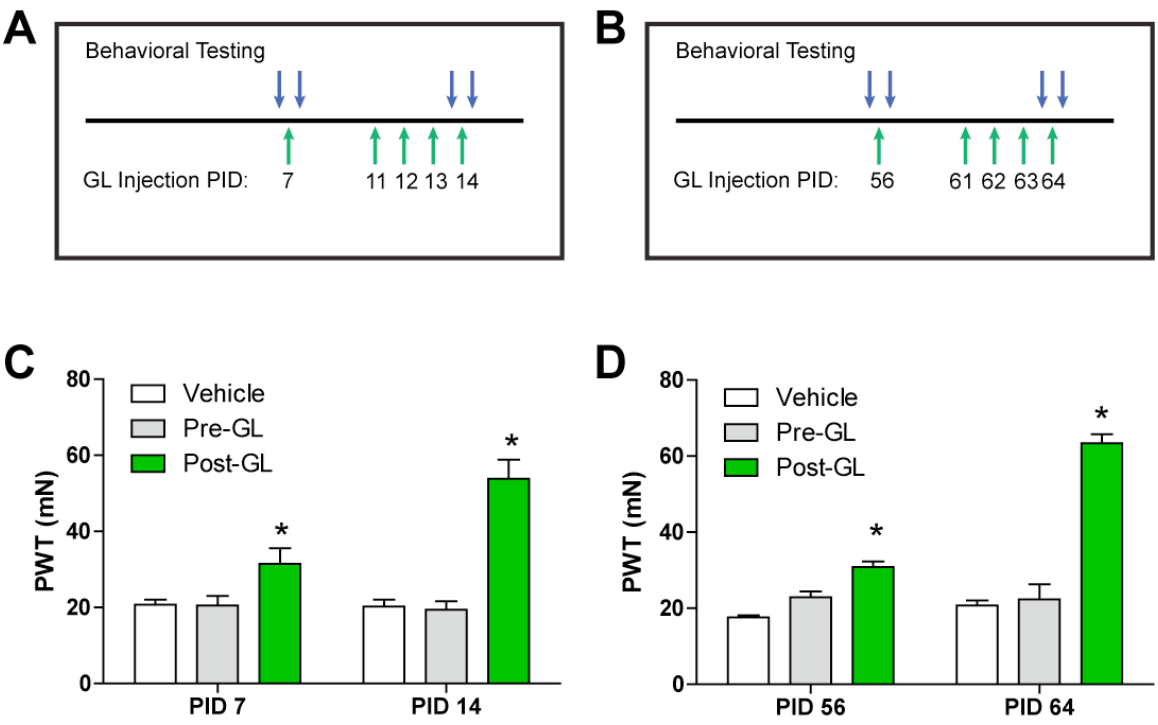


Figure 17: Pre-treatment of glycyrrhizin once daily for 4 days reduces TNI-induced pain hypersensitivity. Glycyrrhizin treatment paradigm. **(A)** Treatment includes a one-time injection of glycyrrhizin (i.p.; 50mg/kg) or treatment with glycyrrhizin one injection per day over four consecutive days. Single injection at PID 7 or one injection per day PID 11-14. Tactile behavior sensitivity was measure before and after injection on PID 7 and 14. **(B)** Single injection at PID 56 or one injection per day PID 61-64. Tactile behavior sensitivity was measured before and after injections on PID 7 and 14. A one-time injection of glycyrrhizin at TNI PID 7 **(C)** or PID 56 **(D)** produced only a partial effect on the paw withdrawal threshold (PWT) (n=6; *P< 0.01, ANOVA, F=19.1, Bonferroni's multiple comparison test. Treatment with glycyrrhizin injections over four consecutive days PID 11-14 **(C)** or PID 61-64 **(D)** successfully reversed TNI decreases in PWT (n=6; *P< 0.01, ANOVA, F=265.9; Bonferroni's multiple comparison test. Data are expressed as mean ± s.e.m.

C. Discussion

Previous studies have implicated HMGB1, a cytokine mediator of inflammation, as having a critical role in neuropathic pain (Chacur *et al.*, 2001; Shibasaki *et al.*, 2010; Otoshi *et al.*, 2011a). In the present investigation, we found that HMGB1 undergoes a chronic subcellular redistribution from the nucleus to the cytoplasm of primary afferent neurons following peripheral nerve injury. Given evidence that cytoplasmic HMGB1 can undergo exocytosis in other cell types (Gardella *et al.*, 2002b; Lee *et al.*, 2010), we further determined that activity can contribute to the release of cytoplasmic HMGB1 from a sensory neuron cell line. We also observed that HMGB1 administration to acutely dissociated primary afferent neurons directly increases the excitability of some sensory neurons. Finally we demonstrated that systemic treatment paradigms using GL, a natural anti-inflammatory triterpene that binds directly to HMGB1, can partially reverse pain behavior at PID 7 and PID 56. Though a single dose of GL can elicit a statistically significant reversal of stimulus dependent pain behavior, a four-day treatment paradigm produces a near complete recovery to pre-injury PWT baseline at PID 14 and PID 64 days. Together these results suggest that HMGB1 has a significant role in sensitizing primary afferent neurons and may contribute to neuropathic pain behavior.

After cellular damage or injury, the subcellular redistribution of HMGB1 from the nucleus to the cytoplasm, following passive and active secretion occurs in a variety of cell types, including neurons (Gardella *et al.*, 2002b; Bonaldi *et al.*, 2003; Lotze & Tracey, 2005; Andersson & Harris, 2010; Lee *et al.*, 2010; Maroso

et al., 2010). Passive secretion of HMGB1 is often a result of cellular damage and occurs instantaneously (Schiraldi *et al.*, 2012). Active secretion occurs in cells undergoing profound stress, such as, exposure to inflammatory mediators, including TNFα, IL-1, and IFN-γ (Wang *et al.*, 1999; Muller *et al.*, 2004). More recent studies in the nervous system demonstrate that when glutamate-exposed primary cortical neurons undergo excitotoxic cell death and secrete HMGB1 (Maroso *et al.*, 2010). Herein, we demonstrated that following peripheral nerve injury or exposure to a depolarizing event, HMGB1 can translocate from the nucleus to the cytoplasm in both sensory neurons and a sensory neuron-like cell line. More importantly, a depolarizing event *in vitro* can elicit neuronal release of HMGB1 into the extracellular environment. Unlike cultured cortical neurons treated with glutamate, this neuronal depolarization did not elicit sensory neuron cell death. Though the mechanisms of HMGB1 release in neurons are largely unknown, cytoplasmic HMGB1 may be further phosphorylated by the PKC and secreted by a calcium-dependent mechanism via CaMKs (Tsung *et al.*, 2007; Muraki *et al.*, 2009).

That HMGB1 can be released by both injured and non-injured sensory neurons suggests a possible influence on nearby neurons, adjacent nerve fibers, and possibly non-neuronal cells in the nervous system. Strong evidence supporting such a signaling event by HMGB1 was discovered by Maroso and colleagues in a chronic epilepsy model. This group elegantly demonstrated that blockade of HMGB1 markedly reduced seizure duration and frequency in rodent cortical neurons (Maroso *et al.*, 2010). Our results herein parallel findings in

cortical neurons in that exposure to HMGB1 can elicit robust states of excitability in primary afferent neurons. These data suggest that HMGB1 is likely to play a modulatory role in ongoing states of peripheral sensitization following nerve injury.

A number of studies have provided evidence of a role of HMGB1 signaling in nervous system pathology following injury within either the peripheral or central nervous system (Mabuchi *et al.*, 2009; Vezzani *et al.*, 2011). Shibasaki and colleagues demonstrated that injection of HMGB1 into the sciatic nerve produced dose-dependent thermal and tactile hyperalgesia (Shibasaki *et al.*, 2010), while direct administration of HMGB1 into the central nervous system by intrathecal route produced robust mechanical hyperalgesia that lasted for up to two hours (O'Connor *et al.*, 2003; Tong *et al.*, 2010). More importantly, multiple exposures to HMGB1 neutralizing antibodies partially reverse SNL-induced mechanical hyperalgesia and bone cancer pain (Shibasaki *et al.*, 2010; Tong *et al.*, 2010). Taken together, it appears that ongoing HMGB1 release after a nerve injury may be a critical factor for the maintenance of neuropathic pain and may be due to a feed-forward regulation state (Faraco *et al.*, 2007).

Like other proinflammatory mediators HMGB1 exhibits both active and passive release (Gardella *et al.*, 2002a; Scaffidi *et al.*, 2002). The basis of these molecular mechanisms that contribute to these release kinetics are largely unknown. That GL is effective after repeated injections suggests that the release of HMGB1 is an ongoing feed-forward mechanism (Gardella *et al.*, 2002b). The presence of extracellular HMGB1 serves to activate HMGB1 receptor activation

that may continue the expression, production and translocation of HMGB1. Since TNI contributes to both nuclear and cytoplasmic HMGB1, it is likely that HMGB1 is undergoing transcription and translation. More importantly, we observed that total HMGB1 protein expression did not change suggesting that production of HMGB1 is ongoing after injury; otherwise a decrease in total protein expression would be evident.

Oral GL is metabolized in the intestine to 18β-glycyrrhetinic acid (GA) and intravenous (i.v.) glycyrrhizin is metabolized into GA when excreted through the bile into the intestines (Ploeger *et al.*, 2001). Both GA and GL are known to directly interact with HMGB1 and inhibit its inflammatory actions in leukocyte chemotaxis, cancer, and post-ischemic liver and brain (Mollica *et al.*, 2007; Sitia *et al.*, 2007; Mabuchi *et al.*, 2009; Ohnishi *et al.*, 2011; Kim *et al.*, 2012; Schiraldi *et al.*, 2012). Interestingly, only the metabolite GA is able to cross the blood-brain barrier (Tabuchi *et al.*, 2012). GA is produced by bacteria in the intestine after oral administration of GL and exhibits a bioavailability of only 1% in plasma (Yamamura *et al.*, 1995). However, GL bioavailability following intraperitoneal administration is estimated to be 65-90%. Given the manner in which we administer the compound, it is unlikely that GL directly impacts neural or non-neural cells in the spinal cord or brain.

HMGB1 neuronal signaling in neuropathic pain may be dependent on either of two receptors, RAGE and/or TLR4. It is known that functional RAGE is present in sensory neurons (Vincent *et al.*, 2007). Shibasaki and colleagues have demonstrated that after SNL, RAGE expression was increased in the primary

afferent neurons, satellite glial cell in the DRG, and Schwann cells in the spinal nerve (Shibasaki *et al.*, 2010). Based on these findings, this group theorized that HMGB1-RAGE signaling might be a promising therapeutic strategy for the management of neuropathic pain. However, the injury-induced release of HMGB1 and its receptor interaction is not restricted to the RAGE as TLR4 is another major receptor of HMGB1 in neuropathic pain models (Kuang *et al.*, 2012).

The characterization of HMGB1-TLR4 interactions has led to the discovery of a cysteine residue at position 106 within HMGB1, which directly binds to TLR4 and induces cytokine release in macrophages (Yang *et al.*, 2010). This same activation of TLR4 site is present on adult cortical neurons and induces heightened excitability in the form of seizure activity (Sayyah *et al.*, 2003; Galic *et al.*, 2008; Maroso *et al.*, 2010). TLR4-dependent neuronal excitability is not limited to the CNS as primary sensory neurons exposed to the endotoxin LPS, the prototypical agonist of TLR4, produces increased excitability (Ochoa-Cortes *et al.*, 2010), concentration-dependent increase in calcium (Qin *et al.*, 2004), inward ion currents and the release of calcitonin gene-related peptide (CGRP) (Diogenes *et al.*, 2011). Subsequently, there is evidence for the central involvement of TLR4 function in both spinal cord inflammation and pain behavior hypersensitivity (Tanga *et al.*, 2004; Tanga *et al.*, 2005; Tong *et al.*, 2010; Kuang *et al.*, 2012).

The subsequent cell signaling function initiated by HMGB1 through its respective receptors may lead to a cascade of metabolic responses (Rauvala &

Rouhiainen, 2007) or the increased production of pro-inflammatory mediators that sustain a chronic inflammatory state (Klune *et al.*, 2008). Further investigation is necessary to elucidate HMGB1 signaling through TLR4 and/or RAGE in the injured PNS, which may reveal novel mechanisms of neuronal HMGB1 activation that contribute to ongoing peripheral sensitization and neuronal hyperexcitability in chronic pain states.

Our present study has definitively found that HMGB1 is actively released and serves as a relevant ligand for the maintenance of neuropathic pain. We have also discovered that ongoing HMGB1 release within the peripheral nervous system contributes to mechanical behavioral hyperalgesia, such that multiple injections of GL (HMGB1 neutralizing agent) effectively attenuated injury-induced mechanical hyperalgesia. Taken together, we believe that HMGB1 can directly alter sensory neuron function and that the ongoing release of HMGB1 in the periphery contributes to neuropathic pain.

CHAPTER IV

TLR4 EXPRESSION AND FUNCTION IN SENSORY NEURONS FOLLOWING PERIPHERAL NERVE INJURY

A. Introduction and hypothesis rationale

Neuropathic pain is a highly complex pain state that involves neuroimmune interactions in both the central and peripheral nervous system. There is increasing evidence that neuropathic pain arising from nerve injury has a central nervous system component that involves spinal glial activation (Watkins & Maier, 2002). Further, TLR4 has been implicated as the mechanistic link between nerve transection, microglia activation, and behavioral hypersensitivity. The involvement of TLR4 function in the generation of neuropathic pain is especially highlighted by observations of both diminished spinal cord inflammation and pain behavior hypersensitivity in rodent lacking TLR4 (Tanga *et al.*, 2004; Tanga *et al.*, 2005). Initial interpretations of these data suggest that elimination or suppression of TLR4 signaling in microglial cells may directly contribute to the nerve injury-induced pain hypersensitivity. However, the injury-induced rodent behavior changes are evident as early as 1 day, while microglial activation elicited by endogenous inflammatory agents is typically delayed until 3 days after the insult (Colburn *et al.*, 1997). The absence of pain behavior in injured TLR4 knockout animals prior to day 3 suggests that the contribution of TLR4 to neuropathic pain may be dependent on cell types other than microglial.

Much of what is known about TLR4 signaling is limited to non-neuronal cells, such as immune, glia, epithelial cells. Only recently has the expression of TLR4 in neurons been definitively characterized (Wadachi & Hargreaves, 2006; Rolls *et al.*, 2007; Acosta & Davies, 2008) and only a few studies have demonstrated its functional role in sensory neurons. For instance, Maroso and colleagues demonstrated that TLR4 activation in cortical neurons results in enhanced seizure activity (Maroso *et al.*, 2010). In sensory neurons, other investigators have found that LPS can directly activate DRG neurons leading to production of inflammatory cytokines by neurons and increased neuronal excitability (Ochoa-Cortes *et al.*, 2010; Diogenes *et al.*, 2011). A very recent finding from White and colleagues, demonstrates that LPS and M3G (TLR4 agonists) administration has the ability to directly enhance sensory neuron excitability by modulating sodium channel function that may contribute to evoked pain hypersensitivity (Due *et al.*, 2012). Interestingly, it has been reported that a low degree of TLR4 activation can promote sensory neuron survival, whereas a high degree of TLR4 activation results in pronounced neuronal dysfunction and death (Nowicki *et al.*, 2010). Collectively, recent studies suggest TLR4 on sensory neurons has a prominent role in neuronal function, but little is known about its function and expression in injured sensory neurons.

In this study, I reasoned that since TLR4 historically functions to generate an immune response and the induction of proinflammatory mediators, then neuronal TLR4 might also serve to alter the functional response of sensory neurons in a neuropathic pain state. Specifically, I explored the hypothesis that

the hyperexcitable state of sensory neurons following a peripheral nerve injury (TNI) is in part attributed to TLR4 in injured DRGs. To test my hypothesis I used immunolabeling and immunoblot techniques, calcium imaging, and pain-related behavior assessment outlined in the previous chapter **(Chapter II)**. Using immunohistochemical methodology, I demonstrated that TLR4 expression decreased in nociceptive neurons after a tibial nerve injury. Moreover, *in vitro,* injured sensory neurons displayed a diminished intracellular calcium response to application of a TLR4 agonist, HMGB1. Finally, unlike intrathecal injections of TLR4 inhibitors, we demonstrated that systemic administration of a small molecule inhibitor for TLR4 did not have an effect on injury-induced tactile hypersensitivity in female rats.

B. Original experimental results

1. Toll-like Receptor 4 is present on sensory neurons

TLR4 expression has been identified in sensory ganglia (Wadachi & Hargreaves, 2006; Ochoa-Cortes *et al.*, 2010). Here, I first examined the extent to which TLR4 protein expression is present in lumbar and trigeminal sensory ganglia *in vivo* **(Figure 18A)**. TLR4 expression present in DRG is localized exclusively in sensory neurons. TLR4-immunopositive sensory neurons exhibit peptidergic and non-peptidergic neuronal phenotypes in the rat lumbar DRG **(Figure 18B-M)**. To characterize a peptidergic sensory neuron subpopulation, TLR4 expression was co-labeled with a CGRP marker. Further to characterize a non-peptidergic sensory neuron population, TLR4 expression was co-labeled

with Isolectin B4 (IB_4). NeuN was used as a neuronal marker. Taken together, TLR4 is present on nociceptive neurons derived from lumbar dorsal root ganglia.

Figure 18:

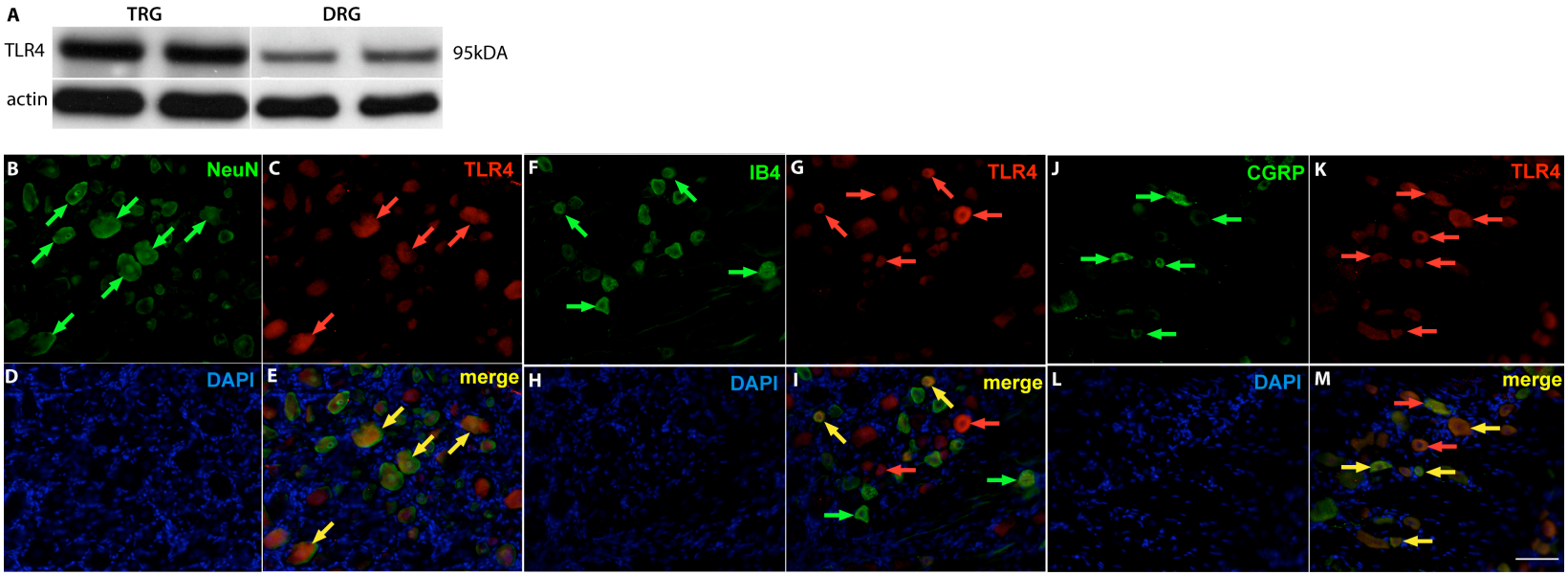


Figure 18: TLR4 expression in sensory neurons. (A) Western blot analysis of TLR4 isolated from trigeminal (TRG) and lumbar $L_{3\text{-}6}$ dorsal root ganglion (DRG) lysates. **(B-M)** Immunofluorescent images of TLR4 in peptidergic and non-peptidergic DRG populations from sectioned naïve L_4 or L_5 DRGs. NeuN is a neuronal marker (**B**, green arrows), which co-labeled with TLR4 (**C**, red arrows). Nuclei are stained with DAPI (**D**, **H**, **L**, blue). Merged images demonstrate co-labeling of NeuN with TLR4 (**E**, yellow arrows). CGRP is a marker for peptidergic containing sensory neurons (**F**, green arrows), which co-labeled with TLR4 (G, red arrows). Merged images demonstrate co-labeling of CGRP containing neurons with TLR4 (**I**, yellow arrows). IB_4 is a marker for nonpeptidergic sensory neurons (**J**, green arrows), which co-labeled with TLR4 (**K**, red arrows). Merged images show co-labeling of IB_4 non-peptidergic sensory neurons with TLR4 (**M**, yellow arrows (n=6; S/D rats) Scale bar, 60μm **(B-M).**

2. Tibial nerve injury induces a decrease in TLR4 protein expression

I next compared the injury-induced effects on TLR4 protein expression in injured $L_{4/5}$ DRGs. TLR4 protein levels in the injured DRGs at PID 3 and 7 exhibited a statistically significant decrease in protein expression when compared with naïve protein levels **(Figure 19A,B)**. Additionally, relative to naïve and sham tissue TLR4 protein expression levels were slightly decreased by TNI at PID 14 **(Figure 20A,B)** but did not demonstrate a significant change at PID 28 **(Figure 20C,D)**. To verify this observation, I investigated the extent to which TLR4 protein expression was decreased in $L_{4/5}$ DRGs ipsilateral to the injury by immunofluorescent imaging **(Figure 21A)**. TLR4 expression was localized primarily in sensory neurons of L_5 DRGs. Following TNI, the percentage of positive TLR4-immunoreactive (IR) sensory neurons decreased when compared to sham-injured animals by 19.6%, (sham: 53.6 ± 2.6, injured: 34.0 ± 2.81) **(Figure 21B)**.

Figure 19:

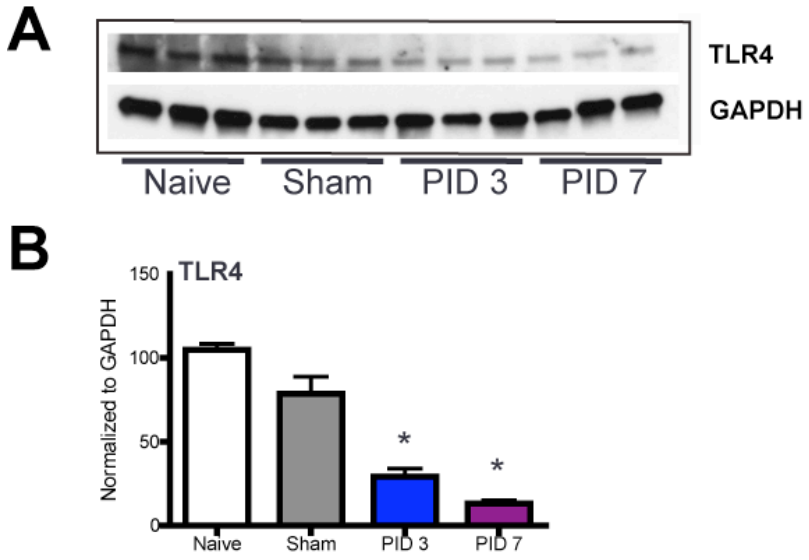


Figure 19: Protein analysis of TLR4 expression following TNI at post injury days 3 and 7. (A) Immunoblot of TLR4 in $L_{4/5}$ DRGs from naïve, sham injured and TNI ipsilateral to the injury at PID 3 and PID 7. GAPDH was used as a loading control to which samples were normalized. **(B)** Representative graph. (n=3 for each group; ANOVA, F=51.34, *P< 0.001, Bonferroni's multiple comparison test).

Figure 20:

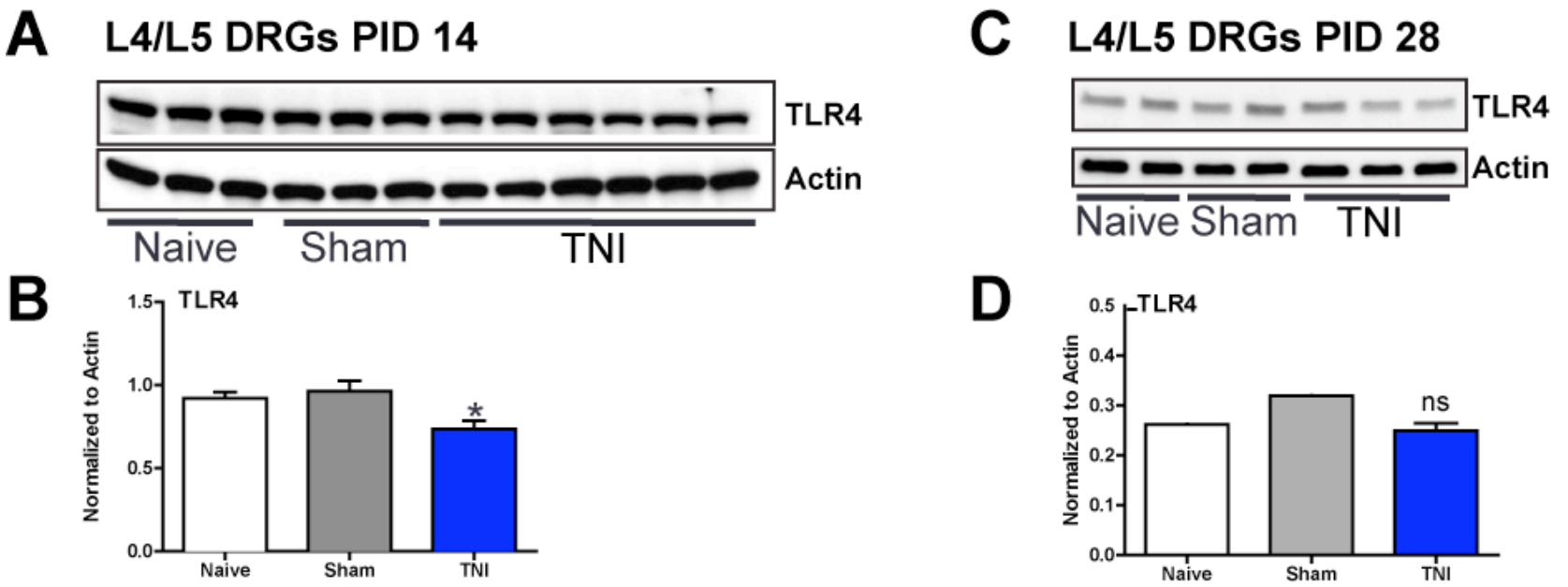


Figure 20: Protein analysis of TLR4 expression following TNI at post injury days 14 and 28. (A,C) Immunoblot of TLR4 in $L_{4/5}$ DRG lysates from naïve, sham, and TNI ipsilateral to the injury at PID 14 and PID 28. Actin was used as a loading control to which samples were normalized. **(B)** Representative graph of TLR4 immunoblot of $L_{4/5}$ lysates PID 14. (n=3 for naïve and sham, n=6 for TNI PID 14; ANOVA, F=5.70, **P*< 0.05, Bonferroni's multiple comparison test). **(D)** Representative graph of TLR4 immunoblot of $L_{4/5}$ lysates PID 28. (n=2 for naïve and sham, n=3 for TNI PID 28; Naïve and sham groups were combined to perform a Student's t-test comparing TNI PID 28; *P*> 0.05,).

Figure 21:

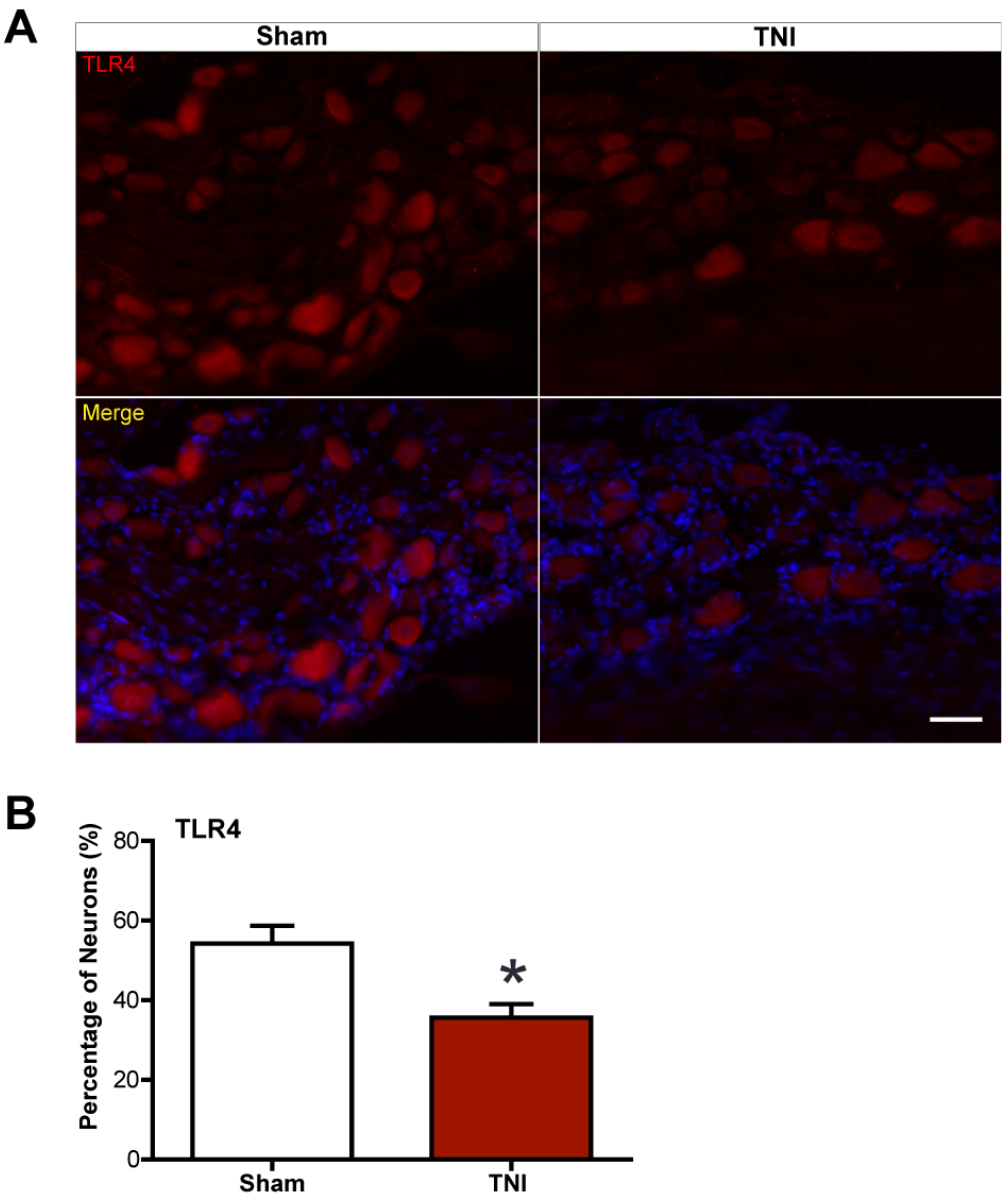


Figure 21: TLR4 immunoreactivity in L_5 DRG primary afferent neurons following TNI in rats. (A) Sections of L_5 DRG stained for TLR4 immunoreactivity are localized primarily to neurons cells in sham-injured and injured L_5 DRGs at PID 14. Scale bar, 50μm. **(B)** Neuronal cell counts were performed on sections of L_5 DRGs derived from sham-injured and TNI at PID 14 animals. Injured L_5 DRGs exhibited a decrease in the percentage of neurons positive for TLR4 compared to sham-injured; sham n=3, PID 14 n=3, Student's t-test, *P< 0.05.

I next evaluated the degree to which a small/medium and large TLR4-immunopositve cells were altered after injury. Cell size was determined by measuring the cross sectional area of each sensory neuron, small and medium diameter neurons were categorized with a cross sectional area of <1000μm^2. Following TNI, the percentage of small and medium diameter TLR4-IR L5 DRGs decreased compared to sham-injured controls (sham: 52.0 ± 2.28, injured: 33.0 ± 2.83) **(Figure 22A,B)**, where as TLR4-IR in large diameter neurons were not altered following injury. Cell counts are shown in **Table 6**. Although the cell count analysis is based on a relatively small total number of sensory neurons accounting for bias in methodology, this initial data collection suggests a possible difference in TLR4 expression in a subset of injured sensory neurons.

Figure 22:

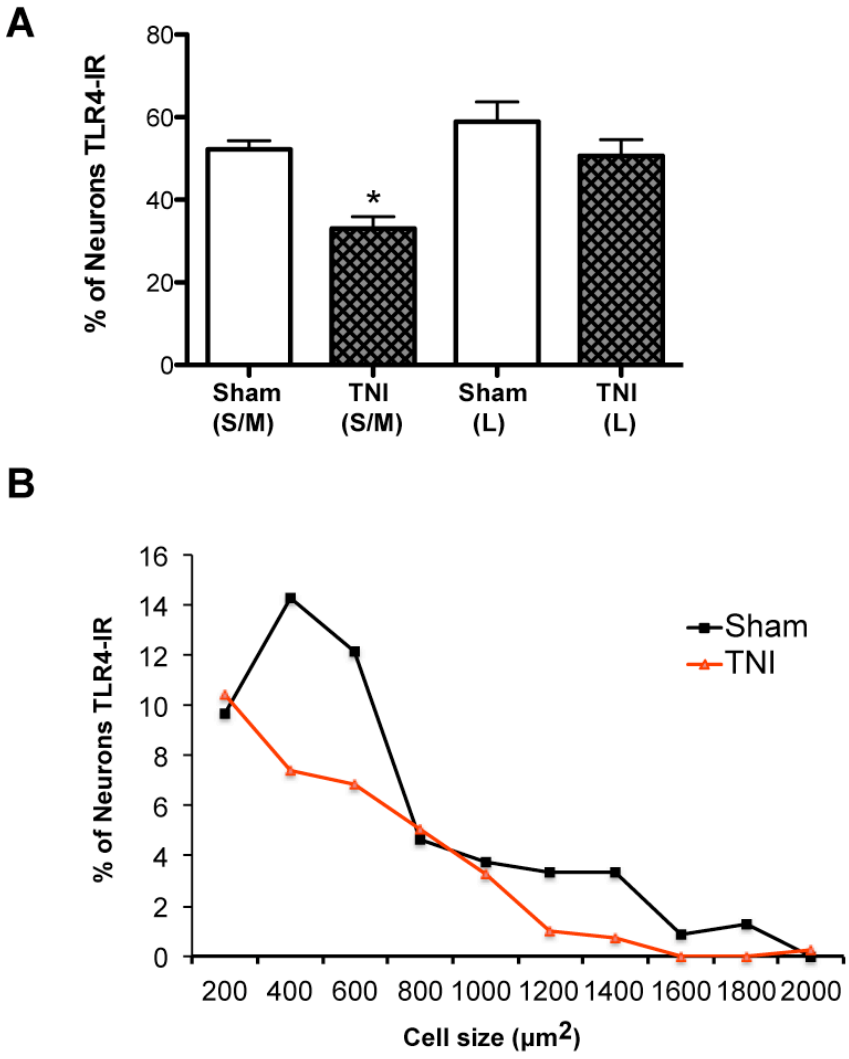


Figure 22: TLR4 immunoreactivity in L_5 DRG small, medium and large primary afferent neurons following TNI in rats. (A) Injured L_5 DRGs exhibited a decrease in the percentage of small/medium (S/M) diameter sensory neurons compared to sham-injured (S/M) neurons at PID 14 (*P< 0.05), unlike large (L) diameter sensory neurons from L_5 DRGs ipsilateral to the injury. Percentage of neurons for each group small/medium, and large represent #TLR4-immunopositive per group/ #neurons per group*100. S/M diameter neurons <1000μm^2. Large diameter neurons > 1000μm^2. (sham n=3, PID 14 n=3, Student's t-test, *P< 0.05). **(B)** Cell size distribution of TLR4-IR in small, medium, large diameter sensory neurons from L_5 DRGs ipsilateral to the injury. The cell distribution is based on 629 neurons (n=6) were categorized as 392 from TNI PID 14 rats, 237 from sham-injured rats. Cell counts are shown in **Table 4**.

Table 6:

	% Small-Medium Neurons (±SEM) (<1000µm2)	% Large Neurons (±SEM) (>1000µm2)	% Total Neurons (±SEM)
Sham (n=3)	52.0 ± 2.28	58.9 ± 4.84	53.6 ± 2.60
TNI (n=3)	33.0 ± 2.83*	50.6 ± 3.94	34.0 ± 2.81*

Table 6: TLR4-immunoreactivity in L_5 DRGs 14 days following tibial nerve Injury. The table represents the values for the data shown in Figure 24 for small, medium, and large DRG neuron cell count analysis. For cell counts in the DRG comparisons were made per animal followed by calculation of group means. Each values is the average mean ± SEM. * denotes statistical significance, $P< 0.05$.

3. TLR4 is present in injured and uninjured neurons after injury

Tibial nerve injury involves complete transection of the tibial nerve that correspond to injured neurons, however uninjured nerves sural, saphenous, common peroneal co-mingle and overlap to an extent innervating adjacent denervated skin (Swett & Woolf, 1985). Injury to the tibial nerve results in injured neurons that lie within the associated L_{4-6} DRGs, which can further release pro-nociceptive mediators in a paracrine manner (e.g. TNFα) that can further act on uninjured sensory neurons to change excitability and function (Sorkin *et al.*, 1997; Schafers *et al.*, 2003). Accordingly, I examined whether TLR4 expression was present on injured and uninjured neurons following TNI, by co-labeling with a well-known marker for neuronal injury, activating transcription factor 3 (ATF3). ATF3 is known for its role in axonal outgrowth (Seijffers *et al.*, 2007) and may function as a negative regulator of TLR signaling in immune cells (Gilchrist *et al.*, 2006). Results from Chapter III, indicate that roughly 31% of sensory neurons express ATF3 after TNI **(Chapter III; Figure 12)**. More importantly, TLR4 co-labeled in both injured (ATF3-IR) and uninjured neurons derived from injured L_5 DRGs **(Figure 23)**.

Figure 23:

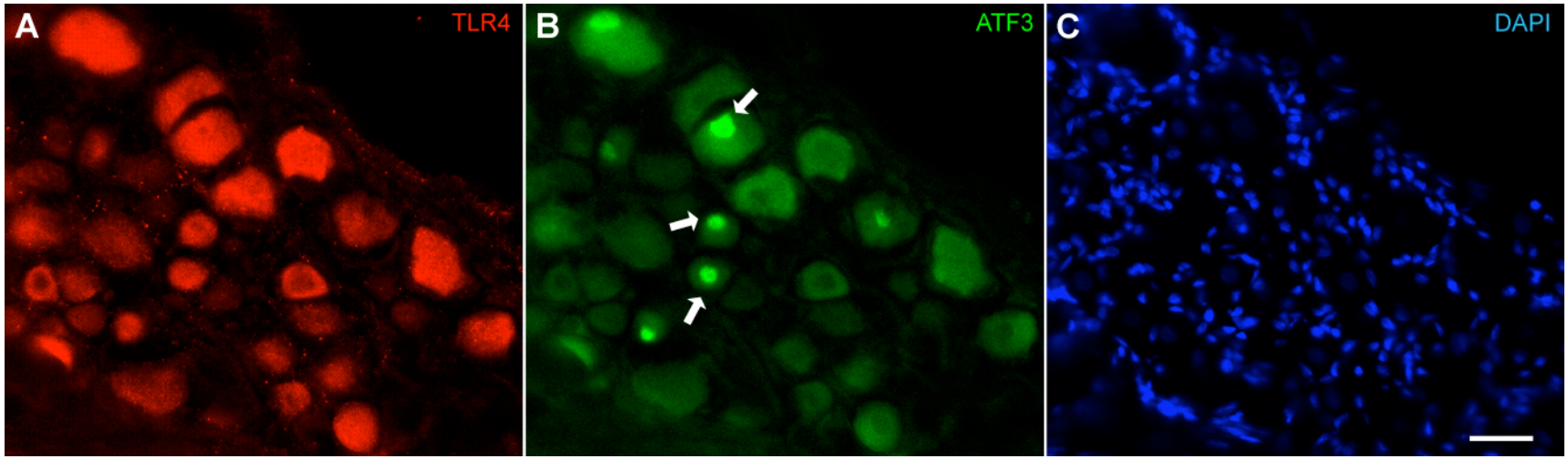


Figure 23: TLR4-immunoreactivity in injured and uninjured in L5 DRG primary afferent neurons. Sectioned L_5 DRGs from TNI at PID 14 stained with **(A)** TLR4 **(B)** ATF3- a marker for neuronal injury **(C)** DAPI- a nuclei marker. White arrows represent colocalization of TLR4 and ATF3 immunoreactivity. TLR4 is present in injured and uninjured neurons. Some injured neurons were negative for TLR4-immunoreactivity. n=3; S/D rats. Scale bar, 30μm.

4. Diminished calcium mobilization by TLR4 endogenous agonist, HMGB1, in dissociated injured sensory neurons

As outlined in Chapter III, HMGB1, an endogenous TLR4 agonist, in naïve dissociated sensory neuron cultures activates calcium mobilization in a number of nociceptive neurons (70%) **(Chapter III; Table 5)**. Using electrophysiological current clamp techniques, under suprathreshold conditions HMGB1 was able to increase the excitability of small and medium diameter neurons in naïve and TNI acutely dissociated sensory neurons (Feldman *et al.*, 2012).

I further examined the extent to which HMGB1 can elicit an intracellular calcium response at multiple time points after a tibial nerve injury. HMGB1 is highly sensitive to the redox environment and cytokine activity is optimal in mildly reduced conditions (Venereau *et al.*, 2012), HMGB1 was supplied in the presence of DTT (reducing agent) and reconstituted in PBS. The same HMGB1 batch was used throughout each experiment and post injury day tested. Following HMGB1 application, capsaicin (TRPV1 agonist) and high K+ (50K) (activates voltage-gated calcium channels) were added to further characterize the phenotype of the imaged cells (**Figure 24**). A response to 50K is indicative of viable sensory neurons, while a response to capsaicin and 50K is characteristic of a nociceptive neuron. Application of HMGB1 to injured dissociated sensory neurons from $L_{4\text{-}5}$ DRGs PID 7, 14, and 28 resulted in a decrease in the percentage of sensory neurons capable of eliciting a calcium response when compared to naïve conditions. Specifically, in the capsaicin-sensitive subpopulation the diminished response was evident at PID 7, 14, and

28 when compared to naïve conditions (X^2[PID 7]= 16.89, X^2[PID 14]= 22.02, X^2[PID 28]= 37.86, *P<0.01). In the non-capsaicin sensitive population, a pronounced decrease in the percentage of neurons that elicited an intracellular calcium flux upon exposure to HMGB1 occurred at PID 28 when compared to naïve conditions (X^2[PID 28]= 12.02, *P<0.01) **(Table 7)**.

Figure 24:

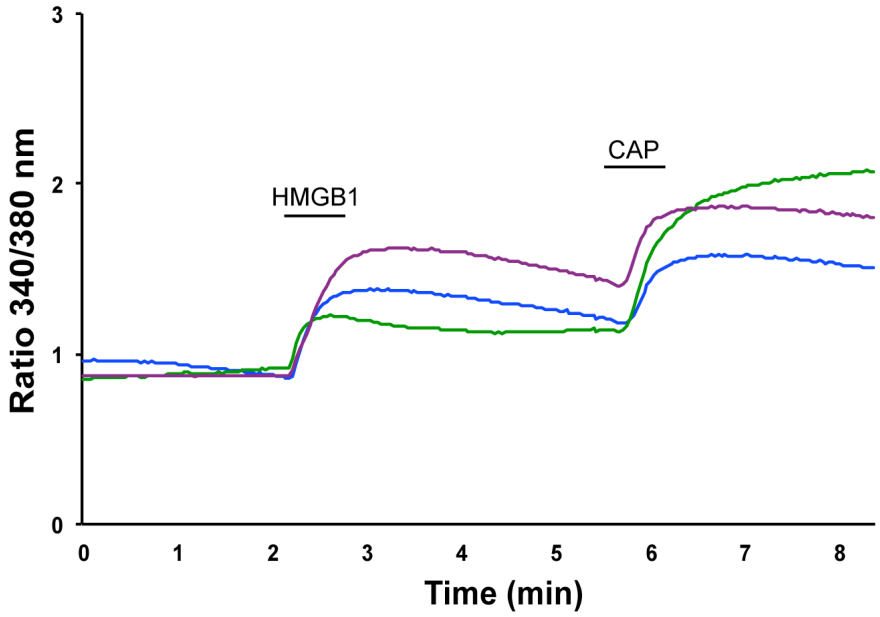


Figure 24: Representative calcium trace in response to the application of HMGB1. The ratio 340/380 (bound/unbound calcium) as a function of time. Each trace represents a single dissociated sensory neuron. HMGB1 was applied directly into the coverslip bathing solution with a final concentration of 0.65µg/ml followed by the application of capsaicin (3nM). Baseline was determined at the beginning of the experiment. A 50% increase over baseline is a positive response to HMGB1 (purple).

Table 7:

	Naïve (%)	PID 7 (%)	PID 14 (%)	PID 28 (%)
Capsaicin-sensitive	70 (31/44)	17 5/28)*	10 (3/29)*	9 (5/56)*
Non-capsaicin-sensitive	19 (26/136)	21 (8/39)#	16 (7/45)^	1(1/75)*

Naïve vs TNI	*
TNI 7 vs TNI 28	#
TNI 14 vs TNI 28	^

Chi-squared text χ^2 with Yates correction
%: (# respond/# total neurons tested for each group)*100

Table 7: Intracellular calcium mobilization is diminished after acute exposure to HMGB1. L_4/L_5 DRGs, ipsilateral to the injury, were dissociated and incubated overnight (~18hrs) in media that did not contain NGF. Sensory neurons were incubated with fura-2AM to visualize calcium mobilization .TNI post injury day (PID) 7, 14, and 28 significantly decreases the percentage of HMGB1 responsive neurons as measured by a change in intracellular calcium influx. * # ^ denotes statistical significance, *P< 0.01. Naïve (n=6); PID 7 (n=4); PID 14 (n=4); PID 28 (n=4). Excitation wavelengths of 340/380nm were measured. Calcium imaging traces that reflected at least a 50% increase over baseline were included in the analysis.

To verify the state of acutely dissociated sensory neurons, I examined the expression of ATF3, in a five-hour and an overnight dissociated sensory neuron preparation. Recently, Ono and colleagues demonstrated that prolonged dissociated sensory neuron cultures induces ATF3 expression and can alter neuronal excitability similar to neuropathic pain states (Ono *et al.*, 2012). ATF3 is known for its role in neurite outgrowth (Seijffers *et al.*, 2006), ability to distinguish injured neurons (Tsujino *et al.*, 2000), and negatively regulating TLR4 signaling (Gilchrist *et al.*, 2006). To determine whether the cultured neurons used in calcium imaging experiment expressed ATF3, L3-L6 were isolated from a single S/D rat and dissociated for either 5hrs or overnight (O/N), ~18hrs. Dissociated sensory neurons were cultured without the presence of NGF. In acutely dissociated cultures (5hrs) ATF3 expression was not present. However, ATF3 was observed in nearly every sensory neuron in O/N cultured conditions. Non-neuronal cells did not express ATF3, suggesting the increase in ATF3 expression is specific to neurons **(Figure 25)**. Taken together, the presence of ATF3 may impact the degree to which HMGB1 may elicit a calcium-mediated response in injured nociceptive neurons.

Figure 25:

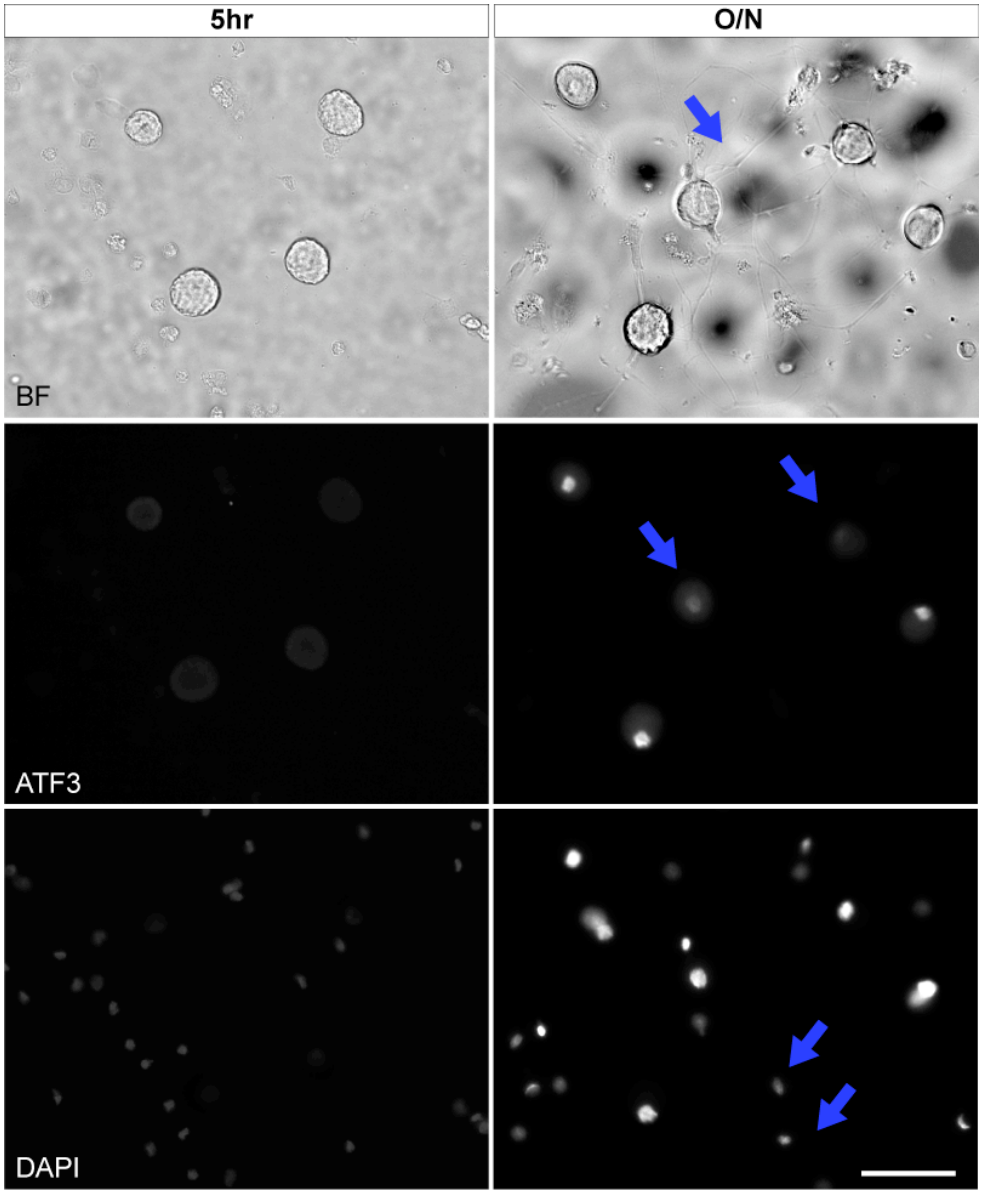


Figure 25: Dissociated primary sensory neuron culture express neuronal injury marker, ATF3. Dissociated primary sensory neurons cultured for 5hrs (left column) and overnight (~18hrs) (right column). Primary sensory neurons cultured overnight begin to express ATF3 (middle panel; blue arrows) and extend neurite processes (top panel; blue arrow). ATF3 is specific to neurons, glia are present in cell cultures as depicted by nuclei-DAPI staining (bottom panel; blue arrows). A single S/D rat was used for this experiment, $L_{3\text{-}6}$ were collected and cultured for 5hr or overnight (O/N) groups, DRGs were cultured without the presence of NGF. Scale bar, 50μm.

5. Systemic administration of TLR4 small molecule inhibitor does not alter pain hypersensitivity in the TNI model of neuropathic pain

Intrathecal administration of TLR4 inhibitors and TLR4 KO animals has been shown to diminish the pain hypersensitivity in neuropathic pain animal models, attributing TLR4 microglia activation in the spinal cord (Tanga *et al.*, 2005; Bettoni *et al.*, 2008; Hutchinson *et al.*, 2008; Wu *et al.*, 2010a). The effect of systemic administration of a TLR4 small molecule inhibitor (compound 15 or HY09) following TNI was tested. HY09 has been shown to disrupt the TLR4/MD-2 complex formation and selectively block TLR4 signal transduction without affecting other homologous TLR family proteins (Bevan *et al.*, 2010). HY09 also demonstrates high specificity and low toxicity *in vitro* and *in vivo* (Wang *et al.*, 2012a), with negligible non-specific inhibitory effects (Chavez *et al.*, 2011). A single injection of HY09 (10mg/kg; i.p.) in baseline uninjured naïve animals did not alter baseline paw withdrawal threshold (PWT). Further a single injection of HY09 (10mg/kg; i.p.) at TNI PID 14 and 28 also did not alter the reduction in PWT **(Figure 26)**.

Figure 26:

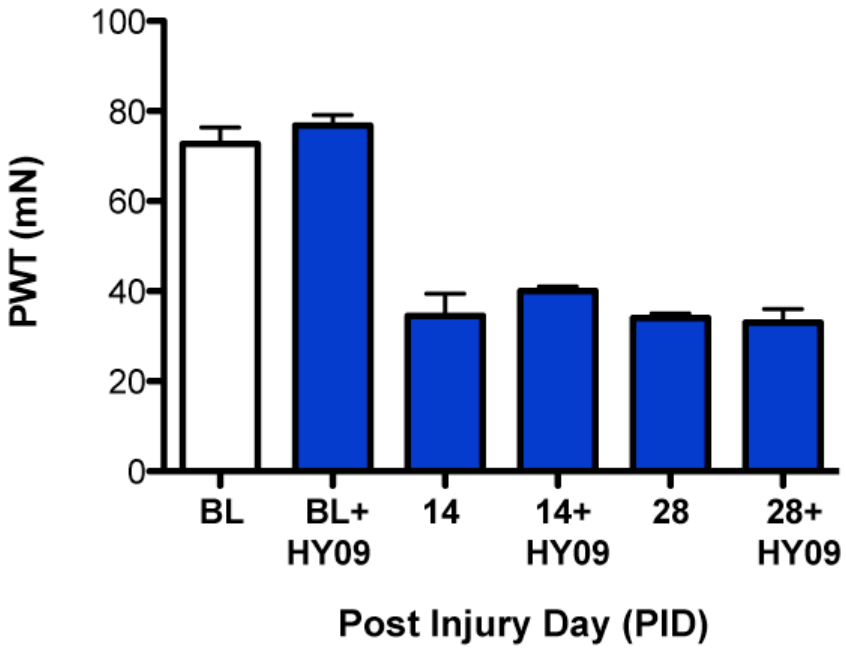


Figure 26: Systemic administration of TLR4 small molecule inhibitor does not alter injury-induced pain hypersensitivity in female rats. A single injection of HY09 (10mg/kg; i.p.) did not have an effect on TNI-induced tactile hyperalgesia at PID 14 and PID 28. HY09 in uninjured animals did not have an effect on effect on tactile sensitivity when compared to each time point PID 14 and 28. PID 14 n=6; PID 28 n=3, female S/D rats. (Student's t-test, *P*> 0.05). Both injections and tactile behavior were performed by Matthew Ripsch. BL: baseline.

C. Discussion

TLR4 has been detected on neuronal and non-neuronal cell types in several pathogenic conditions. Recent reports have begun to elucidate the function role of TLR4 on neurons (Maroso *et al.*, 2010; Ochoa-Cortes *et al.*, 2010; Due *et al.*, 2012). In this present investigation, I found that in the DRG TLR4 is indeed expressed exclusively on sensory neurons. Most notably, TLR4 protein expression in injured ganglia is decreased after a peripheral nerve injury. The results further indicate that small and medium diameter injured sensory neurons may be more susceptible to such a decrease in TLR4 protein expression. Correspondingly, I further observed a diminished calcium response to TLR4 agonist, HMGB1, in injured sensory neurons. We further demonstrated that systemic injections of TLR4/MD2 antagonist in female rats did not reverse injury-induced pain hypersensitivity at PID 14 or 28 suggesting that the TLR4/MD2 interaction may not contribute to chronic injury-induced tactile hypersensitivity. The lack of TLR4 expression and calcium-mediated functional responses may suggest that TLR4 undergoes negative regulation in injured sensory neurons. Taken together, the present investigation suggests that TLR4 expression and function is altered in injured sensory neurons, which may contribute to a neuropathic pain state.

After a peripheral nerve injury, previous literature has demonstrated that TLR4 in the CNS influences the establishment of neuropathic pain. For instance, TLR4 mRNA expression is upregulated primarily on microglia (Lehnardt *et al.*, 2003) and intrathecal injections of TLR4 antagonists can reverse injury-induced

pain hypersensitivity (Tanga *et al.*, 2005; Bettoni *et al.*, 2008; Hutchinson *et al.*, 2008). Neuronal TLR4 either in the spinal cord or dorsal root ganglia is also a prime candidate for perpetuating sensitization, which is a critical component in neuropathic pain states. Neuronal TLR4 expression is found in both central and peripheral nervous system (Tang *et al.*, 2007; Acosta & Davies, 2008; Due *et al.*, 2012). Herein, I demonstrated that unlike in spinal cord, injured dorsal root ganglia decrease TLR4 protein expression. At first glance, a decrease in TLR4 proteins expression suggests termination of downstream signaling or a cessation in TLR4 transcription. Further investigation is necessary to elucidate the precise TLR4 signaling that may transpire in order to account for the decrease in TLR4 protein expression observed in injured DRGs.

Interestingly, TLR4 expression was observed in injured and uninjured neurons by co-labeling with ATF3, a prominent neuronal injury marker (Tsujino *et al.*, 2000). *In vivo* only a fraction of tibial nerve injured DRGs express ATF3, which is comparable to other injury models (Tsujino *et al.*, 2000; Shortland *et al.*, 2006). TLR signaling and ATF3 have been implicated in delayed macrophage recruitment/activation, myelin debris clearance, and axonal regeneration (Seijffers *et al.*, 2006; Boivin *et al.*, 2007). More importantly, ATF3 is involved in suppressing TLR signaling mainly by inhibiting the production of proinflammatory cytokines (Gilchrist *et al.*, 2006; Whitmore *et al.*, 2007). Although, sensory neurons express ATF3 in cultured conditions (Ono *et al.*, 2012) **(Figure 25)**, which may function as a negative regulator of TLR signaling (Gilchrist *et al.*, 2006; Whitmore *et al.*, 2007), TLR4 neuronal activation may persist. In this study,

nearly every dissociated sensory neuron cultured overnight was immunoreactive for ATF3 **(Figure 25)**. Indirect evidence presented demonstrates that *in vitro* sensory neurons acutely exposed to a TLR4 agonist-HMGB1, was capable of eliciting a calcium response. However, injured sensory neurons were observed to have a diminished calcium response to HMGB1, nevertheless, injured neurons still elicited a response.

Furthermore, these correlational studies are consistent with the working hypothesis that TLR4 signaling in injured DRGs may result in distinct neuronal subpopulations. The different subpopulations may depend on the degree of ATF3 mediated TLR4 negative regulation. On one hand TLR4 activation may support axonal regeneration, and on the other hand TLR4 activation may influence calcium regulation/neuronal excitability **(Figure 27)**. Additionally, non-capsaicin sensitive injured sensory neurons displayed an overall latency in diminished calcium response with exposure to HMGB1 **(Table 7)**, suggesting peptidergic and non-peptidergic injured sensory neurons may also have distinct calcium-mediated properties in response to endogenous TLR4 agonists.

The basis for the observed altered calcium response most likely depends on the state of calcium regulation in injured sensory neurons. Other investigators have found that the influx of extracellular calcium through voltage-gated channels (Lirk *et al.*, 2008) and/or depletion of intracellular calcium stores increase neuronal excitability after a peripheral nerve injury (Fuchs *et al.*, 2005; Gemes *et al.*, 2009; Rigaud *et al.*, 2009). In particular, Gemes and colleagues demonstrate a compensatory role of store-operated calcium entry (SOCE) in calcium

homeostasis and functional regulation, which is hypothesized to enhance excitability in injured sensory neurons (Gemes *et al.*, 2011). One recent study has further identified nuclear calcium-CREB signaling in mediating the function of ATF3 activity-dependent transcriptional repression of genes, which are critical for neuronal survival (Zhang *et al.*, 2011). Together with what is known about calcium regulation in injured sensory neurons, suggests that the observed results of diminished calcium response may in fact be a consequence of calcium dysregulation in injured sensory neurons.

Figure 27:

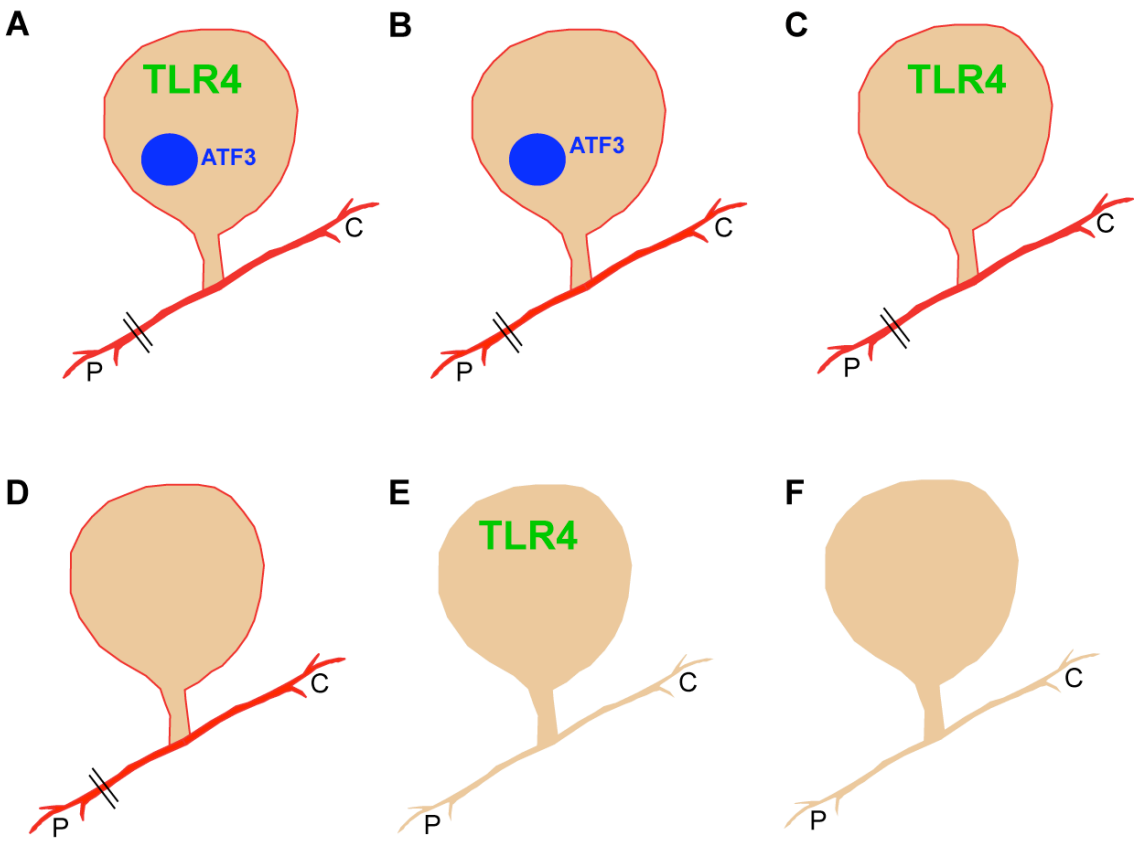


Figure 27: Possible *in vivo* sensory neuron populations after a tibial nerve injury. (A) Injured neuron positive for both TLR4 and ATF3. Negative regulation of TLR4 signaling, involved in axonal regeneration and is less excitable; **(B)** Injured neurons positive for ATF3, no effect on TLR4 signaling; **(C)** Injured neurons positive for TLR4, TLR4 signaling occurs; **(D)** Injured neuron delayed ATF3 expression and not positive for TLR4. In this situation TLR4 activation/degradation rate may outcompete TLR4 transcription, neuron is seen as negative for TLR4; **(E)** Uninjured neuron positive for TLR4, TLR4 signaling occurs; **(F)** Uninjured neuron that does not express TLR4 or ATF3. The red outline depicts an injured sensory neuron. P: peripheral axonal branch, C: central axonal branch, and the double lines correspond to the site of injury.

TLR4 also has the unique ability to signal not only at the plasma membrane (via Myd88-dependent signaling) but also in intracellular endosomal compartments (via TRIF-dependent signaling) to activate NFκB and IFN pro-inflammatory signaling pathway. TLR4 internalization into endosomal compartments can lead to TLR4 lysosomal degradation (Chuang & Ulevitch, 2004), which may account for the partial decrease in TLR4 protein expression evident at PID 3, 7, and 14. Other TLRs (TLR3/7/9) are also known to signal through endolysosomal compartments and agonist stimulation of TLR3/7/9 has recently been shown to sensitize sensory neurons, *in vitro* (Qi *et al.*, 2011). Further investigation is necessary to determine if injured sensory neurons engage in TLR4 signaling at the plasma membrane and intracellular compartments.

TLR4-deficient animals and siRNA have demonstrated the importance of TLR4 in microglia activation in the development of neuropathic pain (Tanga *et al.*, 2005; Wu *et al.*, 2010a). Hutchinson and colleagues provided further evidence that inhibition of TLR4 is also important for well-established neuropathic pain (Hutchinson *et al.*, 2008). The majority of studies examine the central mechanism of TLR4 in neuropathic pain by utilizing TLR4 inhibitors and/or knockdown TLR4 siRNA that are either permeable to the blood brain barrier or administered intrathecally. Herein, we demonstrated that systemic administration of a well-established TLR4/MD2 complex small molecule inhibitor (Bevan *et al.*, 2010; Due *et al.*, 2012) did not prevent injury-induced pain hypersensitivity. Thus, TLR4 in the periphery may have alternative means of activation that is less dependent on

MD2. To date, specific ligand interaction have been structurally identified for only a few exogenous TLR4 agonists, including LPS, morphine, morphine derivate-M3G, which require MD2 (Shimazu *et al.*, 1999; Akashi *et al.*, 2003; Hutchinson *et al.*, 2010a; Lewis *et al.*, 2010; Due *et al.*, 2012). However, under naïve conditions evidence indicates that neurons appear to have minimal MD2 expression (Acosta & Davies, 2008). Thus, the precise mechanism of endogenous TLR4 activation is not known and may require other accessory proteins, such as CD14, for neuronal activation after peripheral nerve injury.

Most recently, Mogil and colleagues have discerned a gender difference in animals after spared nerve injury, such that intrathecal injection of a TLR4 antagonist, LPS-RS was effective in reversing injury-induced mechanical allodynia, but only in male rats (DeLeo & Rutkowski, 2000; Sorge *et al.*, 2011). Similarly, in our studies female rats were subjected to a TNI and a systemic injection of TLR4/MD complex antagonist was not able to reverse injury-induced pain hypersensitivity. Taken together, these findings suggests that in a rodent model of neuropathic pain, female rats may have an alternative TLR4 signaling mechanism, which may dependent on the hormonal environment.

In conclusion, TLR4 expression and function in sensory neurons may be dependent on a number of factors, including TLR4 negative regulation and/or presence of endogenous ligands. Better understanding of these TLR4-mediated signaling events in injured sensory neurons may further provide specific targets for therapeutic interventions for neuropathic pain states without disrupting TLR-mediated inflammatory responses.

CHAPTER V

GENERAL DISCUSSION

A. Overview of results

The major goals of this dissertation were to understand the role of an endogenously secreted cytokine, HMGB1 and to determine the role of one HMGB1-receptor, TLR4 in the dorsal root ganglion in a rodent model of neuropathic pain. A tibial nerve injury model was utilized to examine mechanisms of neuropathic pain. In Chapter II, the tibial nerve injury (TNI) model was characterized on three nociceptive behavior outcomes, thermal hyperalgesia, cold allodynia, and tactile hypersensitivity. Cold allodynia and tactile hypersensitivity was evident soon after the injury and remained thereafter for each experimental time point (> two months). Thermal hyperalgesia was evident beginning at PID 11 lasting for at least 10 days. These results are in line with previous reports (Decosterd & Woolf, 2000; Lee *et al.*, 2000), and establish TNI as an effective model to study a chronic neuropathic pain state.

Chapter III examined the role of HMGB1 in intact and injured sensory neurons and determined if HMGB1 was necessary for TNI-induced pain hypersensitivity. This dissertation found that HMGB1 undergoes subcellular redistribution from the nucleus to the cytoplasm in sensory neurons derived from injured DRGs. In a F11 sensory neuron cell line, HMGB1 was actively released in a feed-forward manner. Furthermore, current-clamp recordings (performed by Dr.

Michael Due) found that exogenous application of HMGB1 can increase neuronal excitability in naïve and injured sensory small and medium sensory neurons. Moreover, calcium imaging demonstrated that exogenous application of HMGB1 can elicit intracellular calcium responses of small and medium naïve and injured sensory neurons. These functional studies involving exogenous application of HMGB1 suggests it may have a direct role in peripheral sensitization. Finally, it was found that systemic administration of glycyrrhizin (GL), an HMGB1 neutralizing agent, can partially reverse injury-induced hypersensitivity; whereas, systemic administration of GL for four consecutive days nearly completely reversed injury-induced pain hypersensitivity. These results indicate that the release of HMGB1 is an ongoing process that may contribute to the development and/or maintenance of neuropathic pain.

Chapter IV examined TLR4 in intact and injured sensory neurons and determined if TLR4 was necessary for TNI-induced pain hypersensitivity. It was first established that TLR4 was expressed primarily in sensory neurons and further co-labeled with peptidergic and non-peptidergic subpopulations in naïve DRGs. Furthermore, after TNI injury TLR4 protein expression decreased from PID3-14, returning to baseline expression by PID 28. The decrease in TLR4 protein expression was found to be restricted to small and medium diameter sensory neurons. Additionally, TLR4 was present on directly injured neurons (ATF3 positive) and uninjured DRG neurons. *In vitro*, it was found that brief exposure of HMGB1, a TLR4 agonist, diminished calcium responses in injured sensory neuron compared to naïve, suggesting a decrease in HMGB1 receptor

signaling. One possibility is injured sensory neurons are influenced by TLR4 negative regulation, which may be the case due to the increased expression of ATF3 (a negative regulator of TLR4 signaling) in injured sensory neurons. Finally, systemic administration of a TLR4/MD2, small molecule inhibitor, did not reverse injury-induced hypersensitivity in female rats. Given that TLR4 may exhibit gender specific issues (Sorge *et al.*, 2011), further studies are needed to determine the precise mechanism of TLR4 activation in injured sensory neurons.

In summary, the presence of HMGB1 and TLR4 in injured DRGs may contribute to a neuropathic pain state. Based on the findings in this dissertation, I am able to draw a number of conclusions: (1) HMGB1 is a dynamic ligand for cytokine function, (2) HMGB1 and TLR4 subcellular localization is context dependent, and (3) there is potential glial bias in neuronal TLR4 activation. Note that HMGB1 signaling is not limited to TLR4 activation, HMGB1 may indeed signal through TLR2, TLR9, and RAGE. The focus of the following discussion is on HMGB1 and TLR4.

B. HMGB1 is a dynamic ligand for cytokine function in sensory neurons

1. Extracellular release

HMGB1 is released as an extracellular cytokine in a number of different disease models (Ohnishi *et al.*, 2011; Kim *et al.*, 2012). These data presented here, in conjunction with Shibasaki and colleagues demonstrate that continuous release of HMGB1 is indeed a contributing factor for neuropathic pain states (Shibasaki *et al.*, 2010) **(Chapter III)**. I presented evidence that cytoplasmic HMGB1 accumulates in the cytoplasm after a peripheral nerve injury and F11 cells release extracellular HMGB1 **(Figure 14,15)**. Moreover, consecutive injections of an HMGB1 neutralizing agent, glycyrrhizin, effectively reversed injury-induced pain hypersensitivity **(Figure 17)**. Recently, glycyrrhizin has been shown to inhibit HMGB1 phosphorylation and secretion (Youn & Shin, 2006; Kim *et al.*, 2012), suggesting that progressive inhibition of HMGB1 extracellular release may dampen pain behavior outcomes. Although phosphorylation and acetylation of HMGB1 mediates active release of HMGB1, the precise mechanism of nuclear translocation is unknown. A recent study examining IL-1β-stimulated release of HMGB1 in cortical astrocytes suggests that a nuclear protein exporter chromosome region maintenance 1 (CRM1), when upregulated, may facilitate the release of HMGB1 (Hayakawa *et al.*, 2010a). Future studies should be directed to understanding nuclear translocation of HMGB1.

2. Extracellular HMGB1 alters neuronal function

Despite evidence of HMGB1 extracellular release by neurons, only a few investigators have explored its role in neuronal function. Recent evidence demonstrates that HMGB1 has a key role in hippocampal function and neuronal excitability in cortical neurons (Maroso *et al.*, 2010; Costello *et al.*, 2011). The data presented in this dissertation provides the first evidence that acute exposure of HMGB1 can alter sensory neuron excitability and function. In naïve conditions, application of HMGB1 resulted in nociceptive neurons to elicit an influx of intracellular calcium and an increase in sensory neuron excitability **(Chapter III; Table 3)**. However injured sensory neurons exposed to HMGB1 increased neuronal excitability in small and medium sensory neurons (Feldman *et al.*, 2012); but displayed an overall diminished calcium response **(Table 5)**. Peripheral nerve injury is known to alter calcium regulation by either VGCCs or intracellular calcium stores in injured sensory neurons (Lirk *et al.*, 2008; Gemes *et al.*, 2011). Future experiments should delineate whether HMGB1 changes in calcium responses are mediated through intracellular calcium stores or a calcium influx at the plasma membrane.

Moreover, it is unclear whether the increase in neuronal excitability is mediated directly or indirectly by the transactivation of ion channels. HMGB1-TLR4 having direct effects on sensory neuronal excitation may involve a number of ion channels downstream of TLR4 activation that can contribute to changes in neuropathic pain states. Both work in the White laboratory and others have demonstrated that lipopolysaccharide (LPS) and morphine-3-glucuronide (M3G), (TLR4-MD2 agonists) increase neuronal excitability in cultured sensory neurons

by TRPV1 and sodium channels NaV1.6, 1.7 and 1.9 (Hua *et al.*, 2007; Diogenes *et al.*, 2011; Due *et al.*, 2012). The activation of TLR4 in the dorsal horn can also indirectly influence the purinergic P2X7, ATP gated ion channel (Clark *et al.*, 2010). Additionally, two potassium channels have also been implicated in TLR receptor signaling, inwardly rectifying potassium channel Kir3.1 and large-conductance calcium- and voltage-activated potassium channel MaxiK (Blunck *et al.*, 2001; Seydel *et al.*, 2001; Scheel *et al.*, 2006). For instance, Kir3.1 channel activity at the plasma membrane may regulate TLR4-mediated signal at an early event by facilitating the recruitment of TLR4 into lipid rafts (Jo *et al.*, 2011).

Most recently, HMGB1 was shown to have a direct interaction with the NMDAR- GluN1 and GluN2B subunits, in isolated hippocampal nerve terminals and in a neuroblastoma cell line to facilitate NMDAR opening and NMDAR-mediated activation of intracellular calcium (Maroso *et al.*, 2010; Pedrazzi *et al.*, 2012). Interestingly, NMDAR receptors are found on primary sensory neuron peripheral terminals, central terminals and the soma (Sato *et al.*, 1993; Liu *et al.*, 1994; Carlton *et al.*, 1995; Ma & Hargreaves, 2000). Here I speculate, HMGB1 may modulate NMDAR in injured DRGs, further contributing to the spontaneous activity of primary afferent neurons

An important question that remains unresolved is which HMGB1 receptor(s) or channel interactions at the plasma membrane are most responsible for neuronal excitability and/or calcium-dependent changes in injured sensory neurons. To distinguish which receptors alter sensory neuron function following a nerve injury, utilizing specific receptor antagonists alone or in

combination to inhibit HMGB1 receptor interaction may provide insight to specific HMGB1-related maladaptive changes in primary sensory neurons.

3. Feed-forward regulation

A working hypothesis states that HMGB1 establishes pro-inflammatory feed-forward cycle that sustains HMGB1-mediated activation of the immune response leading to tissue damage (Faraco *et al.*, 2007). To test this hypothesis preliminary experiments were performed, such that, differentiated F11 cells were exposed to HMGB1 at various concentrations. The application of HMGB1 resulted in the nuclear translocation of HMGB1 into the cytoplasm in F11-neuronal cells, suggesting that extracellular HMGB1 exposure is sufficient to alter its intracellular localization **(Appendix; Figure 29)**. Additionally, evidence presented here demonstrates that following a peripheral never injury the level of HMGB1 protein expression remains; however, the subcellular distribution of HMGB1 is subsequently localized to the cytoplasm in injured DRGs **(Figure 11,14)**. Collectively these findings support the notion that transcription of HMGB1 is ongoing and released into the cytoplasm in injured DRGs.

HMGB1 transcription is facilitated by a strong TATA-less promoter with a repressor element upstream and an enhancer element, which also contains two oestrogen response elements (Borrmann *et al.*, 2001; Lum & Lee, 2001; Nagatani *et al.*, 2001). HMGB1 is under the control tumor suppressor p53- which down regulates promoter activity, whereas, p73α upregulates promoter activity (Uramoto *et al.*, 2003). Krüppel-like factor 4 (KLF4) is a novel transcription factor that has two binding sites on the HMGB1 promoter and is also capable of

regulating transcription of HMGB1 and translocation of HMGB1 in response to LPS (Liu *et al.*, 2008). Whether HMGB1 transcription differs according to cell-type or environmental context is unknown.

At the same time, the release of HMGB1 may regulate gene transcription of its known receptor TLR4. For example, effector molecules downstream of HMGB1 receptor activation, such as ERK, p38 and NFκB signal transduction can further regulate TLR4 transcription (An *et al.*, 2002). TLR4 gene contains consensus-binding sites for Ets family transcription factors, octamer-binding factors, and a composite interferon response factor/Ets motif (Rehli *et al.*, 2000; Roger *et al.*, 2005; Bondeva *et al.*, 2007).

One possibility to prevent feed-forward signaling of HMGB1 is through the negative regulation of HMGB1 receptor signaling. In support of this notion, ATF3 negative regulation has been mathematically predicted to help discriminate between transient and persistent TLR4-induced signals (Litvak *et al.*, 2009). As a negative regulator, ATF3 can act by blocking NFκB binding sites (Gilchrist *et al.*, 2006; Whitmore *et al.*, 2007). In part ATF3 negative regulation may explain the contradictory findings presented in this thesis. I presented data that injured sensory neurons were less responsive to calcium mobilization upon exposure to HMGB1 **(Table 5)**; where as, a brief exposure of HMGB1 increased neuronal excitability in injured sensory neurons **(Chapter IV)**. A caveat to these findings is that nearly all sensory neurons cultured overnight express ATF3 **(Figure 25)** (Ono *et al.*, 2012). Collectively, I speculate that prolonged ATF3 expression in

cultured injured sensory neurons may in fact negatively regulate HMGB1-TLR4 signaling by possibly altering calcium-dependent neuronal function.

4. Bioavailability of HMGB1-receptor activation

Like most proteins, HMGB1 can undergo posttranslational modifications to alter its cytokine bioavailability and functional response. Acetylation, methylation, and riboslylation of HMGB1 are characteristic posttranslational modifications that regulate secretion of HMGB1 in activated or necrotic immune cells. Moreover, recent evidence support that the redox properties of HMGB1 functions to promote inflammatory pathways and chemotaxis (Venereau *et al.*, 2012). The experiments presented in the study utilized a reduced form of HMGB1, however the limitation to these experiments is the unknown variable of the precise redox state of HMGB1 prior to application **(Table 5,7)**. Close examination of the post-translational modifications and corresponding bioactivity of HMGB1 are essential for future studies given the diverse functions of HMGB1 **(Table 2)**.

During inflammation, the predominant form of HMGB1 is C106 thiol (reduced) and disulfide bond C23-45, however during inflammatory resolution HMGB1 is inactive active with a terminally oxidized cysteine residues (Antoine *et al.*, 2010; Yang *et al.*, 2011). Since TLR4 binds HMGB1 specifically at C106 thiol (reduced), this emphasizes the importance of HMGB1 bioavailability. RAGE is the other receptor that has been specifically identified to bind HMGB1 **(Chapter 1; Figure 4)** and it has recently been shown than in a completely reduced environment HMGB1-RAGE interaction can promote cytotoxicity in cancer cells (Tang *et al.*, 2010a; Tang *et al.*, 2010b) **(Table 2)**. Future studies should be

directed towards manipulating the molecular properties of HMGB1 to attain specific biological activity.

A candidate for HMGB1 redox regulation is apurinic/apyrimidinic endonuclease 1/Redox factor-1 (APE1), functions as a redox factor to regulate inflammatory responses and neuronal survival (Vasko *et al.*, 2005). Recently, APE1 has been shown to regulate the release of extracellular HMGB1 and cytokine production (Yuk *et al.*, 2009). The redox state of HMGB1, dictates its' ability of inducing cytokine production to promote inflammation or chemotaxsis (Venereau *et al.*, 2012). However, the redox state of HMGB1 after a peripheral nerve injury is unknown; thereby, I speculate that after a peripheral nerve injury, the redox state of HMGB1 may be distinct from the redox state of HMGB1 during inflammation.

Another factor that may impact the bioavailability of HMGB1 is the presence of HMGB1 decoy receptors. HMGB1 receptor RAGE can exist in a truncated form that lacks the intracellular signaling domain (dominant negative receptor) either as an endogenous secretory splice variant of RAGE (esRAGE) (Yonekura *et al.*, 2003) or soluble RAGE (sRAGE), which is formed when the RAGE receptor is cleaved at the plasma membrane by metalloprotease 10, ADAM 10 (Raucci *et al.*, 2008). Both of which are present in plasma and neutralize the effects of HMGB1, further providing a level of feedback regulation to prevent HMGB1 receptor signaling. For instance, rheumatoid arthritis patients display lower blood levels of sRAGE compared to health controls and non-inflammatory joint disease patients suggesting the lack of inhibition by sRAGE

may maintain HMGB1-receptor interaction (Pullerits *et al.*, 2005). Whether or not plasma levels of sRAGE and esRAGE are altered after a peripheral nerve injury is unknown.

5. HMGB1 functions as a protein complex

It is believed that HMGB1 alone actually lacks cytokine function and instead acts as an extracellular molecular chaperone to facilitate proinflammatory responses (Hreggvidsdottir *et al.*, 2009; Tsan, 2011). Evidence suggests that HMGB1 binds with LPS and enhances LPS-TLR4 mediated TNFα proinflammatory response (Youn *et al.*, 2008). IL-1β bound to HMGB1 has also been suggested to enhance proinflammatory activity (Sha *et al.*, 2008). Additionally, when HMGB1 is in complex it can promote activation of the partner receptor (Hreggvidsdottir *et al.*, 2011). For instance, the reduced form of HMGB1 interacts with SDF1 to promote monocyte migration through the SDF receptor, chemokine (C-X-C motif) receptor (CXCR) 4 not TLR4 (Campana *et al.*, 2009; Schiraldi *et al.*, 2012). Although reports suggest that the co-clustering of CXCR4 and TLR4 is crucial for TLR4 signaling (Kishore *et al.*, 2005; Triantafilou *et al.*, 2008), it is equally possible that the redox state of HMGB1 complexed to partner molecules can further influence cytokine production and leukocyte recruitment following injury or tissue damage (Venereau *et al.*, 2012).

A proteomic analysis has revealed putative proteins that bind with HMGB1. From this analysis one interesting protein Rab10, an endosomal trafficking protein and positive regulator of TLR4, was found to associate with HMGB1 (Hreggvidsdottir *et al.*, 2009; Lee *et al.*, 2010). Understanding the physiological

properties of HMGB1 on its own or as a multi-protein complex will be critical to interpret its role in different disease pathologies.

6. HMGB1-TLR4 interaction at the plasma membrane

The majority of studies have examined TLR4 activation at the plasma membrane by LPS activation. Less is known about the interaction of endogenous ligands with TLR4, CD14 and MD2. It is believed that LPS-MD2-CD14-TLR4 forms a tertiary complex to promote signaling. Unlike MD2, CD14 is not always necessary for LPS mediated signaling (Shimazu *et al.*, 1999; Akashi *et al.*, 2003). MD2 is also important for other exogenous TLR4 agonists, such as M3G and morphine. It is also known that RP105 a dominant negative TLR4 homologue binds to myeloid differentiation protein (MD) 1 and may inhibit TLR4/MD2 signaling (Divanovic *et al.*, 2005). There is minimal evidence suggesting HMGB1-mediated TLR4 activation requires CD14 or MD2, although HMGB1 demonstrates the ability to bind to TLR4/MD2 complex in macrophages (Yang *et al.*, 2010). Recent findings suggest that MD2 mainly recognizes exogenous PAMPs and is capable of discriminating between PAMPs and DAMPs; whereas, CD14 serves as a universal adaptor for both PAMPs and DAMPs (Chun & Seong, 2010).

The question remains as to which accessory proteins, if any, are required for HMGB1-mediated TLR4 signaling in sensory neurons. Interestingly, dorsal root ganglia (DRG) neurons express CD14 and MD1 and have a minimal expression of MD2. It has been demonstrated that TLR4 can form a complex with MD1 (Acosta & Davies, 2008), and it is believed that this combination mediates

non-canonical TLR4 signaling in neurons (Okun *et al.*, 2011). To further suggest this possibility, neurons stimulated with LPS do not translocate NFκB to the nucleus, transcribe IFN-β or activate c-Jun N-terminal kinases (JNK) (Tang *et al.*, 2007). TLR4 has preferential ligand interaction, for instance HSP60 (TLR4 agonist) binds specifically to TLR4 on microglia not neurons, *in vitro*. (Lehnardt *et al.*, 2003; Lehnardt *et al.*, 2008). Here we tested the possibility whether TLR4/MD2 in the periphery contributes to injury induced pain hypersensitivity. Results in this dissertation demonstrate that a specific TLR4/MD2 antagonist injected systemically was not able to reverse injury-induced pain hypersensitivity **(Figure 26)**. From these observations, I speculate that after peripheral nerve injury TLR4 signaling may involve non-canonical receptor activation.

C. HMGB1 and TLR4 subcellular localization is context dependent

1. Subcellular localization of HMGB1

Most cytokines are externalized through the classical endoplasmic reticulum (ER)-golgi exocytotic route. However, HMGB1 lacks an ER localization sequences and is released most likely by secretory lysosomes following immune cell activation (Gardella *et al.*, 2002a). Secretory lysosomes are specific to cells derived from a haematopietic lineage, such as leukocytes and platelets, and generally display features of both lysosomes and secretory granules. Neurons do not contain secretory lysosomes but do contain typical endocytic organelles, early endosomes, late endosomes and lysosomes. However, in injured neurons cytoplasmic HMGB1 is diffusely spread throughout the entire cytosol (Bonaldi *et al.*, 2003; Maroso *et al.*, 2010). It is unclear whether cytoplasmic HMGB1 is

released directly from the cytoplasm or whether cytoplasmic HMGB1 is able to engage with endocytic pathways in neurons.

Presented in this thesis, cytoplasmic HMGB1 is localized throughout the morphology of injured sensory neurons and thus has the possibility to localize to intracellular organelles **(Figure 11)**. A preliminary observation demonstrates that after exposure of HMGB1 to F11 cells, TLR4 accumulates in the cytoplasm **(see Appendix; Figure 30)**. Though it has been previous suggested that HMGB1 may internalize with TLR4 complex after cell surface binding (Yang *et al.*, 2010), whether HMGB1 can traffic to intracellular organelles upon cellular damage or injury in neurons is unknown.

2. TLR4 regulation is dependent of adaptor proteins and negative regulation

TLR4 is the only TLR that signals at the plasma membrane and in intracellular compartments. Upon activation TLR4 can engage in endocytic pathways via clathrin-mediated endocytosis (Latz *et al.*, 2002; Husebye *et al.*, 2006; Tanimura *et al.*, 2008; Wang *et al.*, 2012b). In neurons, Tang and colleagues first demonstrated that TLR4 is localized to both the cell surface and cytosolic compartments (Tang *et al.*, 2007). Further, preliminary observations presented here demonstrate that brief exposure to HMGB1 results in distinct TLR4 localization to the cytosol **(see Appendix; Figure 30)**. However, it is unknown whether TLR4 trafficking in sensory neurons is involved in receptor recycling and/or lysosomal degradation.

Intracellular TLR4 signaling is highly regulated and engages TRIF-dependent pathways resulting in the late NFκB and IRF3 pathway **(Figure 5)**. In fact, TLR4-TRIF dependent signaling is terminated by TAG binding to TRIP preventing downstream IRF3 signaling, which occurs in the late endosome (Palsson-McDermott *et al.*, 2009). Another possible mechanism to inhibit TLR4 signaling is by intracellular negative regulation. For instance, Rab7 is a negative regulator of TLR4 signaling by promoting the translocation of TLR4 to lysosomes for degradation (Wang *et al.*, 2007). On the other hand, Rab10 appears to be a positive regulator of TLR4 by regulating the trafficking rate of TLR4 to the plasma membrane (Wang *et al.*, 2010). Interestingly, I demonstrated a partial decrease in total TLR4 protein expression after tibial nerve injury (PID 3-14), and a decrease in immunoreactivity of TLR4 in small and medium diameter neurons at PID 14 **(Figures 19-22)**. The decrease in protein expression may be a result of TLR4 receptor degradation by Rab trafficking proteins; however, it is equally possible that transcription of TLR4 is decreased after injury. However, the decrease of TLR4 protein expression in injured DRGs was transient, lasting for two weeks post injury.

The question remains whether TLR4 signaling or activation is altered in injured DRGs at a time point beyond two weeks. One possible mechanism may involve negative regulation of TLR4 signaling. For instance, SARM1 (SAM and an Armadillo repeat motif (ARM) 1) is a unique negative regulator of TLR4. It is highly expressed in neurons and inhibits TRIF-dependent signaling. Recent evidence suggests that SARM1 in axons actively promote their own destruction

after injury (Osterloh *et al.*, 2012). Another prominent negative regulator is Tollip, which may inhibit TLR4 or IRAKs (Zhang & Ghosh, 2002) and regulate intracellular trafficking (Yamakami *et al.*, 2003; Ohnuma *et al.*, 2005; Brissoni *et al.*, 2006). However, most evidence for TLR4 negative regulation was examined in non-neuronal cells. Findings presented in this dissertation, provides evidence of a specific neuronal TLR4 negative regulator, ATF3, following peripheral nerve injury **(Figure 12,23)**. *In vitro* experiments demonstrate that dissociated sensory neurons cultured overnight express ATF3 and injured sensory neurons display a diminished calcium response to HMGB1, suggesting that the level of ATF3 may contribute to the degree of HMGB1 elicited calcium mobilization in injured sensory neurons **(Figure 25, Table 7)**. Taken together, I speculate that the negative regulation of TLR4 signaling in injured sensory neurons may contribute to calcium dysregulation and neuronal excitability. Future experiments should focus on determining which negative regulators are present in injured sensory neurons and how TLR4 negative regulators may influence neuronal function after a peripheral nerve injury.

3. Glia bias in neuronal TLR4 activation

Neuropathic pain state involves both the central nervous system and peripheral nervous system. Accumulating evidence has demonstrated a major difference in TLR4 activation may depend on the cell type, either neuronal or non-neuronal. This difference is evident within the central nervous system. Lehnardt and colleagues were unsuccessful in labeling neurons derived from the spinal cord, with TLR4 agonist HSP60 (Lehnardt *et al.*, 2003; Lehnardt *et al.*,

2008). Furthermore, LPS exposure did not promote a typical TLR4 proinflammatory response in spinal neurons, unlike astrocytes and microglia (Faraco *et al.*, 2007; Tang *et al.*, 2007). Thus, I speculate that in sensory neurons TLR4 activation may be distinct from microglia.

HMGB1-TLR4 activation in glia cells may contribute to neuronal function in a paracrine manner. For instance, after chronic constriction injury, TLR4-deficient animals display diminished injury induced pain hypersensitivity. Initial analysis of these data suggested that the pain behavior attenuation was due to the absence of TLR4-dependent microglial and astrocytic activation in the spinal cord contributing to neuronal central sensitization (Tanga *et al.*, 2004; Tanga *et al.*, 2005). In addition, Shibasaki and colleagues and evidence in this dissertation demonstrate that HMGB1 is expressed in satellite glial cells (Shibasaki *et al.*, 2010) **(Figure 11)**, which surround sensory neurons in the DRG, and may also influence neuronal function in a paracrine manner. I further presented evidence that after TNI, HMGB1 was also present in SGC before and after peripheral nerve injury **(Figure 11)**.

Furthermore, it is not known whether HMGB1 is found in synaptic vesicles. However, given what is known about HMGB1 subcellular localization, it is less likely that HMGB1 is released from central primary afferent terminals. Instead, the source of extracellular HMGB1 in the spinal cord is most likely glial cells or interneurons (Kim *et al.*, 2006). Moreover, the recent finding suggesting that HMGB1 can interact with NMDA receptors (Pedrazzi *et al.*, 2012) supports the

notion that HMGB1 released by glia cells or interneurons may influence neuronal function at the level of the spinal cord.

D. Unconventional cytokines

HMGB1 is a dynamic protein that influences transcription in the nucleus and functions as an important alarmin to signal tissue damage. Other alarmins may function in a similar manner as HMGB1. A new candidate, hepatoma derived growth factor (HDGF) has similar biochemical features to that of HMGB1. For instance, HDGF has two nuclear localization signals and shares 32% amino-acid sequence homology with HMGB1 (Zhou *et al.*, 2004). Additionally, HDGF not only stimulates growth in several cell types, but can also act as a neurotrophic factor for neurons (Marubuchi *et al.*, 2006). HDGF has also been shown to display similar nuclear localization and secretion characteristics as HMGB1 (Abouzied *et al.*, 2004). Many alarmins, including HMGB1, are leaderless cytokine proteins that generally are not trafficked through classical ER and Golgi secretory pathways (Nickel & Rabouille, 2009). In inflammatory cells, HMGB1 is trafficked from the nucleus to secretory lysosomes and multivesicular endosomes that fuse with the plasma membrane (Scaffidi *et al.*, 2002; Liu *et al.*, 2006). Similarly, the leaderless cytokine galectin 1 (Gal1) also involves direct translocation from the cytoplasm across the plasma membrane, bypassing classical endocytotic pathways (McGraw *et al.*, 2004; McGraw *et al.*, 2005). Some leadless cytokines, such as HMGB1, IL-1β, migration inhibitory factor (MIF) are oxidation sensitive than may require free cysteine residues for their

bioactivity (Harris & Raucci, 2006). Thus, a reducing environment and the presences of extracellular thiols may promote pathological bioactivity. Taken together, many alarmins may function as cytokines that may undergo unconventional secretion from the cell and respond to various cues from the environment.

E. HMGB1 release in pathological conditions

The release of extracellular HMGB1 reflects the cellular condition. Necrotic cells or inflammatory cells trigger HMGB1 release to signal tissue injury and initiate the inflammatory response or wound repair (Wang *et al.*, 1999; Scaffidi *et al.*, 2002). On the other hand, during apoptosis HMGB1 is retained in the nuclei and does not initiate inflammation in apoptotic cells (Bustin, 2002). Bianchi and colleagues further revealed that by preventing histone H2B serine 14 phosphorylation, this inhibits HMGB1 chromatin interaction in the nucleus and thereby can generate an inflammatory response in apoptotic cells (Scaffidi *et al.*, 2002). In many pathological conditions the HMGB1 cellular condition may change over time. For instance, a neuropathic pain state may involve an inflammatory, repair, and cell death HMGB1 response in both neuronal and non-neuronal cell types. Furthermore, the extracellular release of HMGB1 is not necessarily unique to a neuropathic pain state. Other neurodegenerative conditions, such as Alzheimer's disease and multiple sclerosis, may involve HMGB1 release by activating an inflammatory response, immune and neuronal cells. Overall, HMGB1 is most likely released locally and may function as a

neuroinflammatory factor contributing to neuronal death, microglia activation, and neuronal degeneration (Kim *et al.*, 2006; Faraco *et al.*, 2007; Gao *et al.*, 2011; Kim *et al.*, 2011). However, the underlying cellular condition over time may lead to alternative downstream signaling events that contribute to the role of HMGB1 in specific disease pathology.

F. Proposed mechanism

The precise mechanism of HMGB1-mediated TLR4 signaling in neurons is not known, determining interaction with accessory proteins, adaptor proteins, negative regulators will be critical for determining the maladaptive state of injured sensory neurons. My working hypothesis is as follows: Depending on the sensory neuron phenotype and redox environment, HMGB1 preferentially binds TLR4 at the plasma membrane that (1) may influence neuronal excitability/calcium regulation at the membrane, (2) engage in endocytic pathways contributing to the maintenance of pro-nociceptive cytokines/mediators following peripheral nerve injury. **Figure 30** summarizes my working hypothesis regarding the potential mechanisms of HMGB1 signaling in injured sensory neurons that may contribute to chronic neuropathic pain states.

Figure 28:

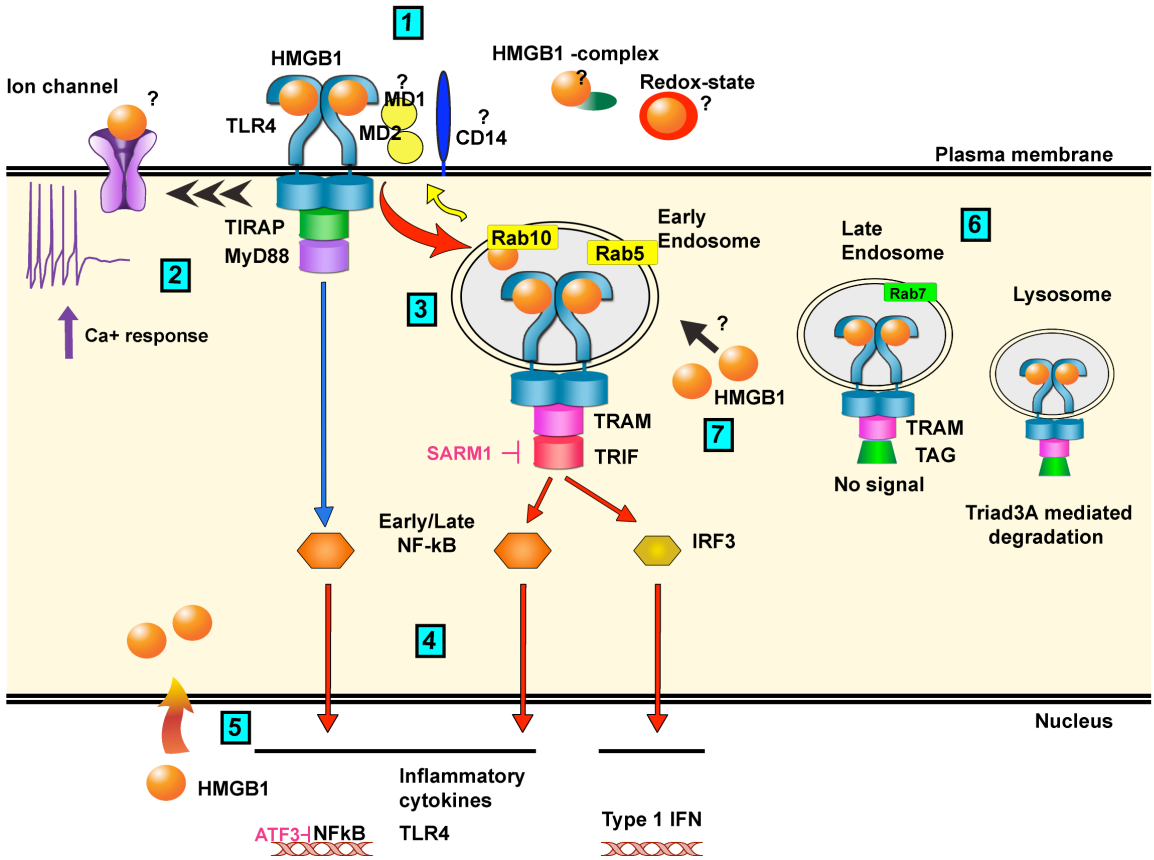

Injured Sensory Neuron
1
HMGB1
HMGB1 -complex
Ion channel
?
MD1
MD2
TLR4
CD14
Redox-state
Plasma membrane
TIRAP
MyD88
Rab10
Rab5
Early Endosome
Late Endosome
6
Rab7
Lysosome
2
3
Ca+ response
HMGB1
TRAM
SARM1
TRIF
7
TRAM
TAG
No signal
Triad3A mediated degradation
Early/Late NF-kB
IRF3
4
5
Nucleus
HMGB1
Inflammatory cytokines
ATF3
NFkB
TLR4
Type 1 IFN

Figure 28: Proposed mechanism in injured sensory neurons. (1) Extracellular release of HMGB1 in injured neuron binds to TLR4. HMGB1 may bind in particular redox state, or in complex. Accessory proteins, MD1, and/or CD14, less likely MD2, are involved in receptor activation. **(2)** Upon TLR4 activation, TIRAP and MyD88 adaptor proteins are recruited. MyD88-dependent signaling activates early NFκB downstream signaling. HMGB1-TLR4 activation at the plasma membrane engages transactivation of ion channels to transiently alter neuronal excitability and function. HMGB1 may interact directly with ion channels. **(3)** Alternatively, HMGB1-TLR4 activation may recruit adaptor protein TRAM/TRIF resulting in the internalization of the HMGB1-TLR4 complex into early endosomes- (Rab5-early endosome marker). TRIF-dependent signaling activates late NFκB and IRF3 downstream signaling. Rab10 facilitates replenishment of TLR4 to the plasma membrane. **(4)** IRF3 downstream signaling results in the production of IFN-β. NFκB downstream signaling results in the induction of pro-inflammatory cytokines (i.e. TNFα, IL-6, IL-1, MCP1) and HMGB1, TLR4 transcription/translation. **(5)** Feed-forward regulation of HMGB1 release **(6)** HMGB1-TLR4 complex is trafficked to late endosomes (Rab7-late endosome marker). TAG is involved in terminating TLR4 downstream signaling by Triad3A mediated degradation. Potential neuronal negative regulators of TLR4 signaling, including but not limited to Rab7, SARM1, ATF3 **(7)** Cytoplasmic HMGB1 may also traffic to endosomal compartments.

G. Summary

The aims of this dissertation were to understand the role of physiologically relevant endogenous ligand HMGB1 and cognate receptor -TLR4 in a tibial nerve injury model of chronic neuropathic pain. In summary the findings of this dissertation show:

- HMGB1 undergoes a subcellular redistribution from the nucleus to the cytoplasm in sensory neurons following peripheral nerve injury. Further, a sensory neuron -surrogate cell line can release HMGB1 into the extracellular environment.

- Direct exposure to HMGB1 increases neuronal excitability in injured and intact sensory neurons. However, *in vitro* there was a diminished calcium response in the presence of exogenous HMGB1 in injured sensory neurons cultured overnight; these observations suggest extracellular HMGB1 may contribute to sensitization of sensory neurons.

- Chronic systemic administration of a HMGB1 neutralizing agent effectively reverses injury-induced tactile hyperalgesia.

- TLR4 protein expression is decreased in injured dorsal root ganglia early in the development of TNI-induced hyperalgesia. More specifically, the decrease of TLR4 protein expression was specific to small and medium diameter sensory neurons.

- Systemic administration of a small molecule TLR4/MD2 inhibitor does not reverse TNI-induced tactile hyperalgesia in female rats.

In conclusion, HMGB1 is a relevant endogenous source in the periphery that contributes to the progression of neuropathic pain states. Endogenous neuronal HMGB1-TLR4 activation is indeed far more complicated and may utilize mechanisms that are distinct from that of the immune system. HMGB1-TLR4 signaling has evolved multiple regulatory mechanisms that may be context dependent. Better understanding HMGB1-mediated signaling events in injured sensory neurons may reveal novel functional roles for immune elements at the level of the nervous system.

APPENDIX

The following is a set of preliminary observations testing whether acute exposure of exogenous HMGB1 can influence endogenous HMGB1 subcellular localization and TLR4 intracellular trafficking.

A. Feed-forward regulation of endogenous HMGB1

A working hypothesis states that HMGB1 establishes pro-inflammatory feed-forward cycle that sustains HMGB1-mediated activation of the immune response leading to tissue damage (Faraco *et al.*, 2007). To test this hypothesis in sensory neurons, I used a differentiated F11 cell line as a surrogate sensory neuron to determine whether exogenous application of HMGB1 could impact the endogenous HMGB1 subcellular localization. In normal conditions, HMGB1 is localized to the nucleus **(Figure 29A)**. Application of exogenous HMGB1 at 0.35µg/ml and 1.4µg/ml was able to promote nuclear subcellular translocation of endogenous HMGB1 into the cytosol **(Figure 29B,C)**. This preliminary observation suggests a possible autonomous feed-forward mechanism that may regulate HMGB1 release.

Figure 29:

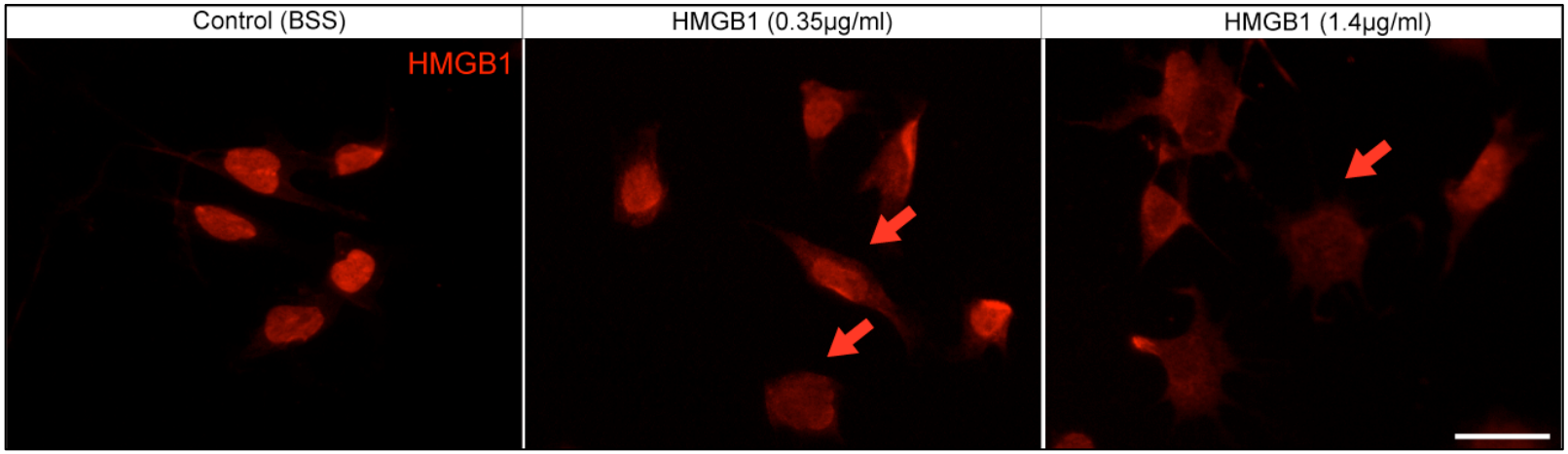


Figure 29: Exogenous HMGB1 induces endogenous HMGB1 nuclear translocation into cytoplasm. HMGB1 was applied to F11 cells at 0.35µg/ml and 1.4µg/ml for 30 mins following cell fixation and labeling with anti-HMGB1 (red). HMGB1 is localized to the nucleus in control conditions, exogenous HMGB1 results in localization throughout the entire morphology of F11 cells (red arrows). A single experiment was performed using 2 wells/coverslips per treatment condition. F11 cells differentiated for 48hrs. Scale bar, 50µm.

B. TLR4 internalization following exposure to exogenous HMGB1

Upon activation TLR4 may undergo internalization into endosomes to facilitate TRIF-dependent signaling (Husebye *et al.*, 2006). To test this phenomenon in sensory neurons, I used a differentiated F11 cell line to determine whether exogenous application of HMGB1 could impact the localization of TLR4 either at the plasma membrane or internalization into intracellular compartments. Differentiated F11 cells express TLR4, 14% of F11 cells (94/668), the signal is spread diffusely throughout the entire morphology of the cell **(Figure 30A)**. Following exposure of HMGB1 for 30 mins, TLR4 localization densely accumulated in the cytoplasm with close proximity to the nucleus **(Figure 30B,C)**. This preliminary observation suggests that TLR4 activation *in vitro* results in receptor internalization in a sensory neuron-like cell line. Taken together, it is hypothesized that sensory neurons are likely to engage in TLR4 signaling at the plasma membrane and through intracellular compartments.

Figure 30:

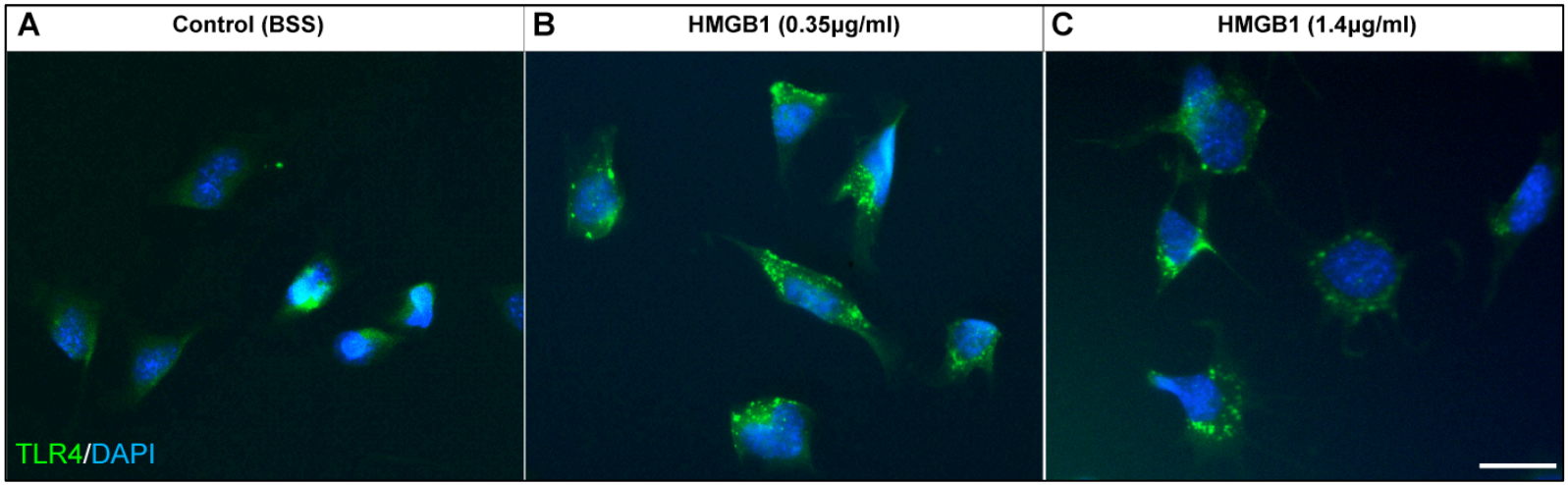


Figure 30: Exogenous application of HMGB1 leads to TLR4 internalization. (A) TLR4-immunoreactivity in F11 cell line. Anti-TLR4(green) and DAPI(blue). **(B,C)** HMGB1 was applied to F11 cells at 0.35µg/ml and 1.4µg/ml for 30 mins following cell fixation and labeling with anti-TLR4(green) and DAPI(blue). Both concentrations resulted in TLR4 accumulation in cytoplasm. Experiment was performed twice. Each experiment used 2 well/coverslips per treatment condition. F11 cells differentiated for 48hrs. Scale bar, 30µm.

REFERENCE LIST

Abbadie, C. (2005) Chemokines, chemokine receptors and pain. *Trends in immunology*, 26, 529-534.

Abdulla, F.A., Stebbing, M.J. & Smith, P.A. (2001) Effects of substance P on excitability and ionic currents of normal and axotomized rat dorsal root ganglion neurons. *Eur J Neurosci*, 13, 545-552.

Abouzied, M.M., Baader, S.L., Dietz, F., Kappler, J., Gieselmann, V. & Franken, S. (2004) Expression patterns and different subcellular localization of the growth factors HDGF (hepatoma-derived growth factor) and HRP-3 (HDGF-related protein-3) suggest functions in addition to their mitogenic activity. *The Biochemical journal*, 378, 169-176.

Acosta, C. & Davies, A. (2008) Bacterial lipopolysaccharide regulates nociceptin expression in sensory neurons. *Journal of neuroscience research*, 86, 1077-1086.

Adachi, O., Kawai, T., Takeda, K., Matsumoto, M., Tsutsui, H., Sakagami, M., Nakanishi, K. & Akira, S. (1998) Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. *Immunity*, 9, 143-150.

Agrawal, A. & Schatz, D.G. (1997) RAG1 and RAG2 form a stable postcleavage synaptic complex with DNA containing signal ends in V(D)J recombination. *Cell*, 89, 43-53.

Agresti, A., Lupo, R., Bianchi, M.E. & Muller, S. (2003) HMGB1 interacts differentially with members of the Rel family of transcription factors. *Biochem Biophys Res Commun*, 302, 421-426.

Akashi, S., Saitoh, S., Wakabayashi, Y., Kikuchi, T., Takamura, N., Nagai, Y., Kusumoto, Y., Fukase, K., Kusumoto, S., Adachi, Y., Kosugi, A. & Miyake, K. (2003) Lipopolysaccharide interaction with cell surface Toll-like receptor 4-MD-2: higher affinity than that with MD-2 or CD14. *J Exp Med*, 198, 1035-1042.

Allen, D.T. & Kiernan, J.A. (1994) Permeation of proteins from the blood into peripheral nerves and ganglia. *Neuroscience*, 59, 755-764.

Alvarez, F.J. & Fyffe, R.E. (2000) Nociceptors for the 21st century. *Current review of pain*, 4, 451-458.

Alvarez, F.J., Morris, H.R. & Priestley, J.V. (1991) Sub-populations of smaller diameter trigeminal primary afferent neurons defined by expression of calcitonin gene-related peptide and the cell surface oligosaccharide recognized by monoclonal antibody LA4. *Journal of neurocytology*, 20, 716-731.

Amir, R., Kocsis, J.D. & Devor, M. (2005) Multiple interacting sites of ectopic spike electrogenesis in primary sensory neurons. *J Neurosci*, 25, 2576-2585.

An, H., Yu, Y., Zhang, M., Xu, H., Qi, R., Yan, X., Liu, S., Wang, W., Guo, Z., Guo, J., Qin, Z. & Cao, X. (2002) Involvement of ERK, p38 and NF-kappaB signal transduction in regulation of TLR2, TLR4 and TLR9 gene expression induced by lipopolysaccharide in mouse dendritic cells. *Immunology*, 106, 38-45.

Anderson, K.V., Bokla, L. & Nusslein-Volhard, C. (1985a) Establishment of dorsal-ventral polarity in the Drosophila embryo: the induction of polarity by the Toll gene product. *Cell*, 42, 791-798.

Anderson, K.V., Jurgens, G. & Nusslein-Volhard, C. (1985b) Establishment of dorsal-ventral polarity in the Drosophila embryo: genetic studies on the role of the Toll gene product. *Cell*, 42, 779-789.

Andersson, U. & Harris, H.E. (2010) The role of HMGB1 in the pathogenesis of rheumatic disease. *Biochimica et biophysica acta*, 1799, 141-148.

Andersson, U., Wang, H., Palmblad, K., Aveberger, A.C., Bloom, O., Erlandsson-Harris, H., Janson, A., Kokkola, R., Zhang, M., Yang, H. & Tracey, K.J. (2000) High mobility group 1 protein (HMG-1) stimulates proinflammatory cytokine synthesis in human monocytes. *J Exp Med*, 192, 565-570.

Antoine, D.J., Williams, D.P., Kipar, A., Laverty, H. & Park, B.K. (2010) Diet restriction inhibits apoptosis and HMGB1 oxidation and promotes inflammatory cell recruitment during acetaminophen hepatotoxicity. *Mol Med*, 16, 479-490.

Arvidsson, J., Ygge, J. & Grant, G. (1986) Cell loss in lumbar dorsal root ganglia and transganglionic degeneration after sciatic nerve resection in the rat. *Brain Res*, 373, 15-21.

Averill, S., McMahon, S.B., Clary, D.O., Reichardt, L.F. & Priestley, J.V. (1995) Immunocytochemical localization of trkA receptors in chemically identified subgroups of adult rat sensory neurons. *Eur J Neurosci*, 7, 1484-1494.

Baba, H., Ji, R.R., Kohno, T., Moore, K.A., Ataka, T., Wakai, A., Okamoto, M. & Woolf, C.J. (2003) Removal of GABAergic inhibition facilitates polysynaptic A fiber-mediated excitatory transmission to the superficial spinal dorsal horn. *Mol Cell Neurosci*, 24, 818-830.

Backonja, M.M. & Stacey, B. (2004) Neuropathic pain symptoms relative to overall pain rating. *J Pain*, 5, 491-497.

Bandell, M., Story, G.M., Hwang, S.W., Viswanath, V., Eid, S.R., Petrus, M.J., Earley, T.J. & Patapoutian, A. (2004) Noxious cold ion channel TRPA1 is activated by pungent compounds and bradykinin. *Neuron*, 41, 849-857.

Baranowski, A.P., Priestley, J.V. & McMahon, S. (1993) Substance P in cutaneous primary sensory neurons--a comparison of models of nerve injury that allow varying degrees of regeneration. *Neuroscience*, 55, 1025-1036.

Baron, R. (2009) Neuropathic pain: a clinical perspective. *Handb Exp Pharmacol*, 3-30.

Barrot, M. (2012) Tests and models of nociception and pain in rodents. *Neuroscience*, 211, 39-50.

Beggs, S. & Salter, M.W. (2007) Stereological and somatotopic analysis of the spinal microglial response to peripheral nerve injury. *Brain Behav Immun*, 21, 624-633.

Beirowski, B., Adalbert, R., Wagner, D., Grumme, D.S., Addicks, K., Ribchester, R.R. & Coleman, M.P. (2005) The progressive nature of Wallerian degeneration in wild-type and slow Wallerian degeneration (WldS) nerves. *BMC neuroscience*, 6, 6.

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

Bender, F.L., Mederos, Y.S.M., Li, Y., Ji, A., Weihe, E., Gudermann, T. & Schafer, M.K. (2005) The temperature-sensitive ion channel TRPV2 is endogenously expressed and functional in the primary sensory cell line F-11. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology*, 15, 183-194.

Bennett, D.L., Boucher, T.J., Armanini, M.P., Poulsen, K.T., Michael, G.J., Priestley, J.V., Phillips, H.S., McMahon, S.B. & Shelton, D.L. (2000) The glial cell line-derived neurotrophic factor family receptor components are differentially regulated within sensory neurons after nerve injury. *J Neurosci*, 20, 427-437.

Bennett, D.L., Michael, G.J., Ramachandran, N., Munson, J.B., Averill, S., Yan, Q., McMahon, S.B. & Priestley, J.V. (1998) A distinct subgroup of small DRG cells express GDNF receptor components and GDNF is protective for these neurons after nerve injury. *J Neurosci*, 18, 3059-3072.

Bennett, G.J. & Xie, Y.K. (1988) A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. *Pain*, 33, 87-107.

Berger, A., Dukes, E.M. & Oster, G. (2004) Clinical characteristics and economic costs of patients with painful neuropathic disorders. *J Pain*, 5, 143-149.

Bertrand, P.P. & Bornstein, J.C. (2002) ATP as a putative sensory mediator: activation of intrinsic sensory neurons of the myenteric plexus via P2X receptors. *J Neurosci*, 22, 4767-4775.

Bessou, P. & Perl, E.R. (1966) Amovement receptor of the small intestine. *The Journal of physiology*, 182, 404-426.

Bettoni, I., Comelli, F., Rossini, C., Granucci, F., Giagnoni, G., Peri, F. & Costa, B. (2008) Glial TLR4 receptor as new target to treat neuropathic pain: efficacy of a new receptor antagonist in a model of peripheral nerve injury in mice. *Glia*, 56, 1312-1319.

Bevan, D.E., Martinko, A.J., Loram, L.C., Stahl, J.A., Taylor, F.R., Joshee, S., Watkins, L.R. & Yin, H. (2010) Selection, Preparation, and Evaluation of Small- Molecule Inhibitors of Toll-Like Receptor 4. *ACS Med Chem Lett*, 1, 194-198.

Bhangoo, S., Ren, D., Miller, R.J., Henry, K.J., Lineswala, J., Hamdouchi, C., Li, B., Monahan, P.E., Chan, D.M., Ripsch, M.S. & White, F.A. (2007a) Delayed functional expression of neuronal chemokine receptors following focal nerve demyelination in the rat: a mechanism for the development of chronic sensitization of peripheral nociceptors. *Molecular pain*, 3, 38.

Bhangoo, S.K., Ren, D., Miller, R.J., Chan, D.M., Ripsch, M.S., Weiss, C., McGinnis, C. & White, F.A. (2007b) CXCR4 chemokine receptor signaling mediates pain hypersensitivity in association with antiretroviral toxic neuropathy. *Brain Behav Immun*, 21, 581-591.

Bianchi, M.E. (2007) DAMPs, PAMPs and alarmins: all we need to know about danger. *J Leukoc Biol*, 81, 1-5.

Bianchi, M.E., Beltrame, M. & Paonessa, G. (1989) Specific recognition of cruciform DNA by nuclear protein HMG1. *Science*, 243, 1056-1059.

Bianchi, M.E. & Manfredi, A. (2004) Chromatin and cell death. *Biochim Biophys Acta*, 1677, 181-186.

Biel, M., Wahl-Schott, C., Michalakis, S. & Zong, X. (2009) Hyperpolarization-activated cation channels: from genes to function. *Physiological reviews*, 89, 847-885.

Bierhaus, A., Haslbeck, K.M., Humpert, P.M., Liliensiek, B., Dehmer, T., Morcos, M., Sayed, A.A., Andrassy, M., Schiekofer, S., Schneider, J.G., Schulz, J.B., Heuss, D., Neundorfer, B., Dierl, S., Huber, J., Tritschler, H., Schmidt, A.M., Schwaninger, M., Haering, H.U., Schleicher, E., Kasper, M., Stern, D.M., Arnold, B. & Nawroth, P.P. (2004) Loss of pain perception in diabetes is dependent on a receptor of the immunoglobulin superfamily. *J Clin Invest*, 114, 1741-1751.

Binder, A., May, D., Baron, R., Maier, C., Tolle, T.R., Treede, R.D., Berthele, A., Faltraco, F., Flor, H., Gierthmuhlen, J., Haenisch, S., Huge, V., Magerl, W., Maihofner, C., Richter, H., Rolke, R., Scherens, A., Uceyler, N., Ufer, M., Wasner, G., Zhu, J. & Cascorbi, I. (2011) Transient receptor potential channel polymorphisms are associated with the somatosensory function in neuropathic pain patients. *PloS one*, 6, e17387.

Birrell, G.J., McQueen, D.S., Iggo, A., Coleman, R.A. & Grubb, B.D. (1991) PGI2-induced activation and sensitization of articular mechanonociceptors. *Neurosci Lett*, 124, 5-8.

Blunck, R., Scheel, O., Muller, M., Brandenburg, K., Seitzer, U. & Seydel, U. (2001) New insights into endotoxin-induced activation of macrophages: involvement of a K+ channel in transmembrane signaling. *J Immunol*, 166, 1009-1015.

Boivin, A., Pineau, I., Barrette, B., Filali, M., Vallieres, N., Rivest, S. & Lacroix, S. (2007) Toll-like receptor signaling is critical for Wallerian degeneration and functional recovery after peripheral nerve injury. *J Neurosci*, 27, 12565-12576.

Boland, L.M. & Dingledine, R. (1990) Expression of sensory neuron antigens by a dorsal root ganglion cell line, F-11. *Brain research. Developmental brain research*, 51, 259-266.

Bonaldi, T., Talamo, F., Scaffidi, P., Ferrera, D., Porto, A., Bachi, A., Rubartelli, A., Agresti, A. & Bianchi, M.E. (2003) Monocytic cells hyperacetylate chromatin protein HMGB1 to redirect it towards secretion. *EMBO J*, 22, 5551-5560.

Bondeva, T., Roger, T. & Wolf, G. (2007) Differential regulation of Toll-like receptor 4 gene expression in renal cells by angiotensin II: dependency on AP1 and PU.1 transcriptional sites. *American journal of nephrology*, 27, 308-314.

Borrmann, L., Kim, I., Schultheiss, D., Rogalla, P. & Bullerdiek, J. (2001) Regulation of the expression of HMG1, a co-activator of the estrogen receptor. *Anticancer research*, 21, 301-305.

Bowman, C.C., Rasley, A., Tranguch, S.L. & Marriott, I. (2003) Cultured astrocytes express toll-like receptors for bacterial products. *Glia*, 43, 281-291.

Boyd, I.A. & Kalu, K.U. (1979) Scaling factor relating conduction velocity and diameter for myelinated afferent nerve fibres in the cat hind limb. *The Journal of physiology*, 289, 277-297.

Bradbury, E.J., Burnstock, G. & McMahon, S.B. (1998) The expression of P2X3 purinoreceptors in sensory neurons: effects of axotomy and glial-derived neurotrophic factor. *Mol Cell Neurosci*, 12, 256-268.

Braz, J.M. & Basbaum, A.I. (2010) Differential ATF3 expression in dorsal root ganglion neurons reveals the profile of primary afferents engaged by diverse noxious chemical stimuli. *Pain*, 150, 290-301.

Brentano, F., Kyburz, D. & Gay, S. (2009) Toll-like receptors and rheumatoid arthritis. *Methods Mol Biol*, 517, 329-343.

Brissoni, B., Agostini, L., Kropf, M., Martinon, F., Swoboda, V., Lippens, S., Everett, H., Aebi, N., Janssens, S., Meylan, E., Felberbaum-Corti, M., Hirling, H., Gruenberg, J., Tschopp, J. & Burns, K. (2006) Intracellular trafficking of interleukin-1 receptor I requires Tollip. *Current biology : CB*, 16, 2265-2270.

Brittain, J.M., Duarte, D.B., Wilson, S.M., Zhu, W., Ballard, C., Johnson, P.L., Liu, N., Xiong, W., Ripsch, M.S., Wang, Y., Fehrenbacher, J.C., Fitz, S.D., Khanna, M., Park, C.K., Schmutzler, B.S., Cheon, B.M., Due, M.R., Brustovetsky, T., Ashpole, N.M., Hudmon, A., Meroueh, S.O., Hingtgen, C.M., Brustovetsky, N., Ji, R.R., Hurley, J.H., Jin, X., Shekhar, A., Xu, X.M., Oxford, G.S., Vasko, M.R., White, F.A. & Khanna, R. (2011) Suppression of inflammatory and neuropathic pain by uncoupling CRMP-2 from the presynaptic Ca(2+) channel complex. *Nature medicine*, 17, 822-829.

Brown, A.G. (1983) Neuronal organization in the dorsal horn of the spinal cord. *Acta morphologica Hungarica*, 31, 87-99.

Brown, A.G., Fyffe, R.E., Rose, P.K. & Snow, P.J. (1981) Spinal cord collaterals from axons of type II slowly adapting units in the cat. *The Journal of physiology*, 316, 469-480.

Brown, M.C., Perry, V.H., Lunn, E.R., Gordon, S. & Heumann, R. (1991) Macrophage dependence of peripheral sensory nerve regeneration: possible involvement of nerve growth factor. *Neuron*, 6, 359-370.

Buchanan, M.M., Hutchinson, M., Watkins, L.R. & Yin, H. (2010) Toll-like receptor 4 in CNS pathologies. *J Neurochem*, 114, 13-27.

Bullitt, E. (1990) Expression of c-fos-like protein as a marker for neuronal activity following noxious stimulation in the rat. *The Journal of comparative neurology*, 296, 517-530.

Burgess, P.R. & Perl, E.R. (1967) Myelinated afferent fibres responding specifically to noxious stimulation of the skin. *The Journal of physiology*, 190, 541-562.

Bustin, M. (2001) Revised nomenclature for high mobility group (HMG) chromosomal proteins. *Trends in biochemical sciences*, 26, 152-153.

Bustin, M. (2002) At the crossroads of necrosis and apoptosis: signaling to multiple cellular targets by HMGB1. *Science's STKE : signal transduction knowledge environment*, 2002, pe39.

Byrod, G., Rydevik, B., Johansson, B.R. & Olmarker, K. (2000) Transport of epidurally applied horseradish peroxidase to the endoneurial space of dorsal root ganglia: a light and electron microscopic study. *Journal of the peripheral nervous system : JPNS*, 5, 218-226.

Cain, D.M., Khasabov, S.G. & Simone, D.A. (2001) Response properties of mechanoreceptors and nociceptors in mouse glabrous skin: an in vivo study. *J Neurophysiol*, 85, 1561-1574.

Calogero, S., Grassi, F., Aguzzi, A., Voigtlander, T., Ferrier, P., Ferrari, S. & Bianchi, M.E. (1999) The lack of chromosomal protein Hmg1 does not disrupt cell growth but causes lethal hypoglycaemia in newborn mice. *Nat Genet*, 22, 276-280.

Campana, L., Bosurgi, L., Bianchi, M.E., Manfredi, A.A. & Rovere-Querini, P. (2009) Requirement of HMGB1 for stromal cell-derived factor-1/CXCL12-dependent migration of macrophages and dendritic cells. *Journal of leukocyte biology*, 86, 609-615.

Campbell, J.N. & Meyer, R.A. (2006) Mechanisms of neuropathic pain. *Neuron*, 52, 77-92.

Campbell, J.N., Raja, S.N., Meyer, R.A. & Mackinnon, S.E. (1988) Myelinated afferents signal the hyperalgesia associated with nerve injury. *Pain*, 32, 89-94.

Caramalho, I., Lopes-Carvalho, T., Ostler, D., Zelenay, S., Haury, M. & Demengeot, J. (2003) Regulatory T cells selectively express toll-like receptors and are activated by lipopolysaccharide. *J Exp Med*, 197, 403-411.

Cario, E., Rosenberg, I.M., Brandwein, S.L., Beck, P.L., Reinecker, H.C. & Podolsky, D.K. (2000) Lipopolysaccharide activates distinct signaling pathways in intestinal epithelial cell lines expressing Toll-like receptors. *J Immunol*, 164, 966-972.

Carlton, S.M., Hargett, G.L. & Coggeshall, R.E. (1995) Localization and activation of glutamate receptors in unmyelinated axons of rat glabrous skin. *Neurosci Lett*, 197, 25-28.

Carpentier, P.A., Begolka, W.S., Olson, J.K., Elhofy, A., Karpus, W.J. & Miller, S.D. (2005) Differential activation of astrocytes by innate and adaptive immune stimuli. *Glia*, 49, 360-374.

Casula, M., Iyer, A.M., Spliet, W.G., Anink, J.J., Steentjes, K., Sta, M., Troost, D. & Aronica, E. (2011) Toll-like receptor signaling in amyotrophic lateral sclerosis spinal cord tissue. *Neuroscience*, 179, 233-243.

Caterina, M.J. & Julius, D. (2001) The vanilloid receptor: a molecular gateway to the pain pathway. *Annual review of neuroscience*, 24, 487-517.

Caterina, M.J., Schumacher, M.A., Tominaga, M., Rosen, T.A., Levine, J.D. & Julius, D. (1997) The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature*, 389, 816-824.

Catez, F., Yang, H., Tracey, K.J., Reeves, R., Misteli, T. & Bustin, M. (2004) Network of dynamic interactions between histone H1 and high-mobility-group proteins in chromatin. *Mol Cell Biol*, 24, 4321-4328.

Chacur, M., Milligan, E.D., Gazda, L.S., Armstrong, C., Wang, H., Tracey, K.J., Maier, S.F. & Watkins, L.R. (2001) A new model of sciatic inflammatory neuritis (SIN): induction of unilateral and bilateral mechanical allodynia following acute unilateral peri-sciatic immune activation in rats. *Pain*, 94, 231-244.

Chakravarty, S. & Herkenham, M. (2005) Toll-like receptor 4 on nonhematopoietic cells sustains CNS inflammation during endotoxemia, independent of systemic cytokines. *J Neurosci*, 25, 1788-1796.

Chan, J.K., Roth, J., Oppenheim, J.J., Tracey, K.J., Vogl, T., Feldmann, M., Horwood, N. & Nanchahal, J. (2012) Alarmins: awaiting a clinical response. *J Clin Invest*, 122, 2711-2719.

Chan, J.R., Watkins, T.A., Cosgaya, J.M., Zhang, C., Chen, L., Reichardt, L.F., Shooter, E.M. & Barres, B.A. (2004) NGF controls axonal receptivity to myelination by Schwann cells or oligodendrocytes. *Neuron*, 43, 183-191.

Chavez, S.A., Martinko, A.J., Lau, C., Pham, M.N., Cheng, K., Bevan, D.E., Mollnes, T.E. & Yin, H. (2011) Development of beta-amino alcohol derivatives that inhibit Toll-like receptor 4 mediated inflammatory response as potential antiseptics. *J Med Chem*, 54, 4659-4669.

Chen, G.Y., Tang, J., Zheng, P. & Liu, Y. (2009) CD24 and Siglec-10 selectively repress tissue damage-induced immune responses. *Science*, 323, 1722-1725.

Chen, K.B., Uchida, K., Nakajima, H., Yayama, T., Hirai, T., Rodriguez Guerrero, A., Kobayashi, S., Ma, W.Y., Liu, S.Y., Zhu, P. & Baba, H. (2011) High-mobility group box-1 and its receptors contribute to proinflammatory response in the acute phase of spinal cord injury in rats. *Spine (Phila Pa 1976)*, 36, 2122-2129.

Chien, L.Y., Cheng, J.K., Chu, D., Cheng, C.F. & Tsaur, M.L. (2007) Reduced expression of A-type potassium channels in primary sensory neurons induces mechanical hypersensitivity. *J Neurosci*, 27, 9855-9865.

Choe, J.Y., Crain, B., Wu, S.R. & Corr, M. (2003) Interleukin 1 receptor dependence of serum transferred arthritis can be circumvented by toll-like receptor 4 signaling. *J Exp Med*, 197, 537-542.

Choi, Y., Yoon, Y.W., Na, H.S., Kim, S.H. & Chung, J.M. (1994) Behavioral signs of ongoing pain and cold allodynia in a rat model of neuropathic pain. *Pain*, 59, 369-376.

Chou, D.K., Zhang, J., Smith, F.I., McCaffery, P. & Jungalwala, F.B. (2004) Developmental expression of receptor for advanced glycation end products (RAGE), amphoterin and sulfoglucuronyl (HNK-1) carbohydrate in mouse cerebellum and their role in neurite outgrowth and cell migration. *J Neurochem*, 90, 1389-1401.

Christianson, C.A., Dumlao, D.S., Stokes, J.A., Dennis, E.A., Svensson, C.I., Corr, M. & Yaksh, T.L. (2011) Spinal TLR4 mediates the transition to a persistent mechanical hypersensitivity after the resolution of inflammation in serum-transferred arthritis. *Pain*, 152, 2881-2891.

Chuang, H.H., Prescott, E.D., Kong, H., Shields, S., Jordt, S.E., Basbaum, A.I., Chao, M.V. & Julius, D. (2001) Bradykinin and nerve growth factor release the capsaicin receptor from PtdIns(4,5)P2-mediated inhibition. *Nature*, 411, 957-962.

Chuang, T.H. & Ulevitch, R.J. (2004) Triad3A, an E3 ubiquitin-protein ligase regulating Toll-like receptors. *Nat Immunol*, 5, 495-502.

Chun, K.H. & Seong, S.Y. (2010) CD14 but not MD2 transmit signals from DAMP. *International immunopharmacology*, 10, 98-106.

Chung, M.K. & Caterina, M.J. (2007) TRP channel knockout mice lose their cool. *Neuron*, 54, 345-347.

Clark, A.K., Staniland, A.A., Marchand, F., Kaan, T.K., McMahon, S.B. & Malcangio, M. (2010) P2X7-dependent release of interleukin-1beta and nociception in the spinal cord following lipopolysaccharide. *J Neurosci*, 30, 573-582.

Coggeshall, R.E., Dougherty, P.M., Pover, C.M. & Carlton, S.M. (1993) Is large myelinated fiber loss associated with hyperalgesia in a model of experimental peripheral neuropathy in the rat? *Pain*, 52, 233-242.

Colburn, R.W., DeLeo, J.A., Rickman, A.J., Yeager, M.P., Kwon, P. & Hickey, W.F. (1997) Dissociation of microglial activation and neuropathic pain behaviors following peripheral nerve injury in the rat. *J Neuroimmunol*, 79, 163-175.

Costello, D.A., Watson, M.B., Cowley, T.R., Murphy, N., Murphy Royal, C., Garlanda, C. & Lynch, M.A. (2011) Interleukin-1alpha and HMGB1 mediate hippocampal dysfunction in SIGIRR-deficient mice. *J Neurosci*, 31, 3871-3879.

Costello, E., Saudan, P., Winocour, E., Pizer, L. & Beard, P. (1997) High mobility group chromosomal protein 1 binds to the adeno-associated virus replication protein (Rep) and promotes Rep-mediated site-specific cleavage of DNA, ATPase activity and transcriptional repression. *EMBO J*, 16, 5943-5954.

Costigan, M., Befort, K., Karchewski, L., Griffin, R.S., D'Urso, D., Allchorne, A., Sitarski, J., Mannion, J.W., Pratt, R.E. & Woolf, C.J. (2002) Replicate high-density rat genome oligonucleotide microarrays reveal hundreds of regulated genes in the dorsal root ganglion after peripheral nerve injury. *BMC neuroscience*, 3, 16.

Costigan, M., Belfer, I., Griffin, R.S., Dai, F., Barrett, L.B., Coppola, G., Wu, T., Kiselycznyk, C., Poddar, M., Lu, Y., Diatchenko, L., Smith, S., Cobos, E.J., Zaykin, D., Allchorne, A., Gershon, E., Livneh, J., Shen, P.H., Nikolajsen, L., Karppinen, J., Mannikko, M., Kelempisioti, A., Goldman, D., Maixner, W., Geschwind, D.H., Max, M.B., Seltzer, Z. & Woolf, C.J. (2010) Multiple chronic pain states are associated with a common amino acid-changing allele in KCNS1. *Brain*, 133, 2519-2527.

Costigan, M., Scholz, J. & Woolf, C.J. (2009) Neuropathic pain: a maladaptive response of the nervous system to damage. *Annual review of neuroscience*, 32, 1-32.

Cox, J.J., Reimann, F., Nicholas, A.K., Thornton, G., Roberts, E., Springell, K., Karbani, G., Jafri, H., Mannan, J., Raashid, Y., Al-Gazali, L., Hamamy, H., Valente, E.M., Gorman, S., Williams, R., McHale, D.P., Wood, J.N., Gribble, F.M. & Woods, C.G. (2006) An SCN9A channelopathy causes congenital inability to experience pain. *Nature*, 444, 894-898.

Croze, S., Duclaux, R. & Kenshalo, D.R. (1976) The thermal sensitivity of the polymodal nociceptors in the monkey. *The Journal of physiology*, 263, 539-562.

D'Mello, R. & Dickenson, A.H. (2008) Spinal cord mechanisms of pain. *Br J Anaesth*, 101, 8-16.

Dalal, A., Tata, M., Allegre, G., Gekiere, F., Bons, N. & Albe-Fessard, D. (1999) Spontaneous activity of rat dorsal horn cells in spinal segments of sciatic projection following transection of sciatic nerve or of corresponding dorsal roots. *Neuroscience*, 94, 217-228.

Daston, M.M. & Ratner, N. (1991) Expression of P30, a protein with adhesive properties, in Schwann cells and neurons of the developing and regenerating peripheral nerve. *The Journal of cell biology*, 112, 1229-1239.

De Vry, J., Kuhl, E., Franken-Kunkel, P. & Eckel, G. (2004) Pharmacological characterization of the chronic constriction injury model of neuropathic pain. *Eur J Pharmacol*, 491, 137-148.

Decosterd, I., Ji, R.R., Abdi, S., Tate, S. & Woolf, C.J. (2002) The pattern of expression of the voltage-gated sodium channels Na(v)1.8 and Na(v)1.9 does not change in uninjured primary sensory neurons in experimental neuropathic pain models. *Pain*, 96, 269-277.

Decosterd, I. & Woolf, C.J. (2000) Spared nerve injury: an animal model of persistent peripheral neuropathic pain. *Pain*, 87, 149-158.

Decosterd, I., Woolf, C.J. (2000) Spared nerve injury: an animal model of persistent peripheral neuropathic pain. *Pain*, 87, 149-158.

Degryse, B., Bonaldi, T., Scaffidi, P., Muller, S., Resnati, M., Sanvito, F., Arrigoni, G. & Bianchi, M.E. (2001) The high mobility group (HMG) boxes of the nuclear protein HMG1 induce chemotaxis and cytoskeleton reorganization in rat smooth muscle cells. *The Journal of cell biology*, 152, 1197-1206.

DeLeo, J.A. & Rutkowski, M.D. (2000) Gender differences in rat neuropathic pain sensitivity is dependent on strain. *Neurosci Lett*, 282, 197-199.

Devor, M. (1991) Sensory basis of autotomy in rats. *Pain*, 45, 109-110.

Devor, M. (2006) Response of nerves to injury in relation to neuropathic pain *Textbook of Pain*. Elsevier, pp. 905-927.

Devor, M. (2009) Ectopic discharge in Abeta afferents as a source of neuropathic pain. *Experimental brain research. Experimentelle Hirnforschung. Experimentation cerebrale*, 196, 115-128.

Dib-Hajj, S.D., Cummins, T.R., Black, J.A. & Waxman, S.G. (2010) Sodium channels in normal and pathological pain. *Annual review of neuroscience*, 33, 325-347.

Didierlaurent, A., Brissoni, B., Velin, D., Aebi, N., Tardivel, A., Kaslin, E., Sirard, J.C., Angelov, G., Tschopp, J. & Burns, K. (2006) Tollip regulates proinflammatory responses to interleukin-1 and lipopolysaccharide. *Mol Cell Biol*, 26, 735-742.

Diogenes, A., Ferraz, C.C., Akopian, A.N., Henry, M.A. & Hargreaves, K.M. (2011) LPS sensitizes TRPV1 via activation of TLR4 in trigeminal sensory neurons. *Journal of dental research*, 90, 759-764.

Ditsworth, D., Zong, W.X. & Thompson, C.B. (2007) Activation of poly(ADP)-ribose polymerase (PARP-1) induces release of the pro-inflammatory mediator HMGB1 from the nucleus. *J Biol Chem*, 282, 17845-17854.

Divanovic, S., Trompette, A., Atabani, S.F., Madan, R., Golenbock, D.T., Visintin, A., Finberg, R.W., Tarakhovsky, A., Vogel, S.N., Belkaid, Y., Kurt-Jones, E.A. & Karp, C.L. (2005) Negative regulation of Toll-like receptor 4 signaling by the Toll-like receptor homolog RP105. *Nat Immunol*, 6, 571-578.

Djouhri, L., Fang, X., Koutsikou, S. & Lawson, S.N. (2012) Partial nerve injury induces electrophysiological changes in conducting (uninjured) nociceptive and nonnociceptive DRG neurons: Possible relationships to aspects of peripheral neuropathic pain and paresthesias. *Pain*, 153, 1824-1836.

Djouhri, L. & Lawson, S.N. (2004) Abeta-fiber nociceptive primary afferent neurons: a review of incidence and properties in relation to other afferent A-fiber neurons in mammals. *Brain research. Brain research reviews*, 46, 131-145.

Dodd, J. & Jessell, T.M. (1985) Lactoseries carbohydrates specify subsets of dorsal root ganglion neurons projecting to the superficial dorsal horn of rat spinal cord. *J Neurosci*, 5, 3278-3294.

Doyle, S., Vaidya, S., O'Connell, R., Dadgostar, H., Dempsey, P., Wu, T., Rao, G., Sun, R., Haberland, M., Modlin, R. & Cheng, G. (2002) IRF3 mediates a TLR3/TLR4-specific antiviral gene program. *Immunity*, 17, 251-263.

Dubin, A.E. & Patapoutian, A. (2010) Nociceptors: the sensors of the pain pathway. *J Clin Invest*, 120, 3760-3772.

Due, M.R., Piekarz, A.D., Wilson, N., Feldman, P., Ripsch, M.S., Chavez, S., Yin, H., Khanna, R. & White, F.A. (2012) Neuroexcitatory effects of morphine-3-glucuronide are dependent on Toll-like receptor 4 signaling. *Journal of neuroinflammation*, 9, 200.

Dunne, A. & O'Neill, L.A. (2003) The interleukin-1 receptor/Toll-like receptor superfamily: signal transduction during inflammation and host defense. *Science's STKE : signal transduction knowledge environment*, 2003, re3.

Dworkin, R.H., O'Connor, A.B., Audette, J., Baron, R., Gourlay, G.K., Haanpaa, M.L., Kent, J.L., Krane, E.J., Lebel, A.A., Levy, R.M., Mackey, S.C., Mayer, J., Miaskowski, C., Raja, S.N., Rice, A.S., Schmader, K.E., Stacey, B., Stanos, S., Treede, R.D., Turk, D.C., Walco, G.A. & Wells, C.D. (2010) Recommendations for the pharmacological management of neuropathic pain: an overview and literature update. *Mayo Clinic proceedings. Mayo Clinic*, 85, S3-14.

Ebadi, M., Bashir, R.M., Heidrick, M.L., Hamada, F.M., Refaey, H.E., Hamed, A., Helal, G., Baxi, M.D., Cerutis, D.R. & Lassi, N.K. (1997) Neurotrophins and their receptors in nerve injury and repair. *Neurochemistry international*, 30, 347-374.

El Gazzar, M., Yoza, B.K., Chen, X., Garcia, B.A., Young, N.L. & McCall, C.E. (2009) Chromatin-specific remodeling by HMGB1 and linker histone H1 silences proinflammatory genes during endotoxin tolerance. *Mol Cell Biol*, 29, 1959-1971.

Emery, E.C., Young, G.T., Berrocoso, E.M., Chen, L. & McNaughton, P.A. (2011) HCN2 ion channels play a central role in inflammatory and neuropathic pain. *Science*, 333, 1462-1466.

Fages, C., Nolo, R., Huttunen, H.J., Eskelinen, E. & Rauvala, H. (2000) Regulation of cell migration by amphoterin. *Journal of cell science*, 113 (Pt 4), 611-620.

Fang, H. & Luo, Y. (1996) Effects of nerve growth factor on axonal retrograde transport after axonal injury of motoneurons. *Journal of Tongji Medical University = Tong ji yi ke da xue xue bao*, 16, 27-31.

Fang, X., Djouhri, L., McMullan, S., Berry, C., Waxman, S.G., Okuse, K. & Lawson, S.N. (2006) Intense isolectin-B4 binding in rat dorsal root ganglion neurons distinguishes C-fiber nociceptors with broad action potentials and high Nav1.9 expression. *J Neurosci*, 26, 7281-7292.

Fang, X., McMullan, S., Lawson, S.N. & Djouhri, L. (2005) Electrophysiological differences between nociceptive and non-nociceptive dorsal root ganglion neurones in the rat in vivo. *The Journal of physiology*, 565, 927-943.

Faraco, G., Fossati, S., Bianchi, M.E., Patrone, M., Pedrazzi, M., Sparatore, B., Moroni, F. & Chiarugi, A. (2007) High mobility group box 1 protein is released by neural cells upon different stresses and worsens ischemic neurodegeneration in vitro and in vivo. *Journal of neurochemistry*, 103, 590-603.

Feldman, P., Due, M.R., Ripsch, M.S., Khanna, R. & White, F.A. (2012) The persistent release of HMGB1 contributes to tactile hyperalgesia in a rodent model of neuropathic pain. *Journal of neuroinflammation*, 9, 180.

Ferri, G.L., Sabani, A., Abelli, L., Polak, J.M., Dahl, D. & Portier, M.M. (1990) Neuronal intermediate filaments in rat dorsal root ganglia: differential distribution of peripherin and neurofilament protein immunoreactivity and effect of capsaicin. *Brain Res*, 515, 331-335.

Finnerup, N.B., Sindrup, S.H. & Jensen, T.S. (2010) The evidence for pharmacological treatment of neuropathic pain. *Pain*, 150, 573-581.

Follenfant, R.L., Nakamura-Craig, M., Henderson, B. & Higgs, G.A. (1989) Inhibition by neuropeptides of interleukin-1 beta-induced, prostaglandin-independent hyperalgesia. *British journal of pharmacology*, 98, 41-43.

Foreman, R.D. (1999) Mechanisms of cardiac pain. *Annual review of physiology*, 61, 143-167.

Francel, P.C., Harris, K., Smith, M., Fishman, M.C., Dawson, G. & Miller, R.J. (1987) Neurochemical characteristics of a novel dorsal root ganglion X neuroblastoma hybrid cell line, F-11. *J Neurochem*, 48, 1624-1631.

Frantz, S., Kobzik, L., Kim, Y.D., Fukazawa, R., Medzhitov, R., Lee, R.T. & Kelly, R.A. (1999) Toll4 (TLR4) expression in cardiac myocytes in normal and failing myocardium. *J Clin Invest*, 104, 271-280.

Fried, K., Brodin, E. & Theodorsson, E. (1989) Substance P-, CGRP- and NPY-immunoreactive nerve fibers in rat sciatic nerve-end neuromas. *Regulatory peptides*, 25, 11-24.

Fuchs, A., Lirk, P., Stucky, C., Abram, S.E. & Hogan, Q.H. (2005) Painful nerve injury decreases resting cytosolic calcium concentrations in sensory neurons of rats. *Anesthesiology*, 102, 1217-1225.

Fukuoka, H., Kawatani, M., Hisamitsu, T. & Takeshige, C. (1994) Cutaneous hyperalgesia induced by peripheral injection of interleukin-1 beta in the rat. *Brain Res*, 657, 133-140.

Fullmer, J.M., Riedl, M.S., Higgins, L. & Elde, R. (2004) Identification of some lectin IB4 binding proteins in rat dorsal root ganglia. *Neuroreport*, 15, 1705-1709.

Gaillard, C. & Strauss, F. (2000) High affinity binding of proteins HMG1 and HMG2 to semicatenated DNA loops. *BMC molecular biology*, 1, 1.

Galic, M.A., Riazi, K., Heida, J.G., Mouihate, A., Fournier, N.M., Spencer, S.J., Kalynchuk, L.E., Teskey, G.C. & Pittman, Q.J. (2008) Postnatal inflammation increases seizure susceptibility in adult rats. *J Neurosci*, 28, 6904-6913.

Gangloff, M., Weber, A.N., Gibbard, R.J. & Gay, N.J. (2003) Evolutionary relationships, but functional differences, between the Drosophila and human Toll-like receptor families. *Biochemical Society transactions*, 31, 659-663.

Gao, H.M., Zhou, H., Zhang, F., Wilson, B.C., Kam, W. & Hong, J.S. (2011) HMGB1 acts on microglia Mac1 to mediate chronic neuroinflammation that drives progressive neurodegeneration. *J Neurosci*, 31, 1081-1092.

Gardella, S., Andrei, C., Ferrera, D., Lotti, L.V., Torrisi, M.R., Bianchi, M.E. & Rubartelli, A. (2002a) The nuclear protein HMGB1 is secreted by monocytes via a non-classical, vesicle-mediated secretory pathway. *EMBO Rep*, 3, 995-1001.

Gardella, S., Andrei, C., Ferrera, D., Lotti, L.V., Torrisi, M.R., Bianchi, M.E. & Rubartelli, A. (2002b) The nuclear protein HMGB1 is secreted by monocytes via a non-classical, vesicle-mediated secretory pathway. *EMBO reports*, 3, 995-1001.

Garrison, C.J., Dougherty, P.M., Kajander, K.C. & Carlton, S.M. (1991) Staining of glial fibrillary acidic protein (GFAP) in lumbar spinal cord increases following a sciatic nerve constriction injury. *Brain Res*, 565, 1-7.

Gaskin, D.J. & Richard, P. (2012) The economic costs of pain in the United States. *J Pain*, 13, 715-724.

Geber, C., Baumgartner, U., Schwab, R., Muller, H., Stoeter, P., Dieterich, M., Sommer, C., Birklein, F. & Treede, R.D. (2009) Revised definition of neuropathic pain and its grading system: an open case series illustrating its use in clinical practice. *The American journal of medicine*, 122, S3-12.

Gemes, G., Bangaru, M.L., Wu, H.E., Tang, Q., Weihrauch, D., Koopmeiners, A.S., Cruikshank, J.M., Kwok, W.M. & Hogan, Q.H. (2011) Store-operated Ca2+ entry in sensory neurons: functional role and the effect of painful nerve injury. *J Neurosci*, 31, 3536-3549.

Gemes, G., Rigaud, M., Weyker, P.D., Abram, S.E., Weihrauch, D., Poroli, M., Zoga, V. & Hogan, Q.H. (2009) Depletion of calcium stores in injured sensory neurons: anatomic and functional correlates. *Anesthesiology*, 111, 393-405.

Georgel, P., Jiang, Z., Kunz, S., Janssen, E., Mols, J., Hoebe, K., Bahram, S., Oldstone, M.B. & Beutler, B. (2007) Vesicular stomatitis virus glycoprotein G activates a specific antiviral Toll-like receptor 4-dependent pathway. *Virology*, 362, 304-313.

Gerke, M.B. & Plenderleith, M.B. (2001) Binding sites for the plant lectin Bandeiraea simplicifolia I-isolectin B(4) are expressed by nociceptive primary sensory neurones. *Brain Res*, 911, 101-104.

Giancotti, V., Bandiera, A., Sindici, C., Perissin, L. & Crane-Robinson, C. (1996) Calcium-dependent ADP-ribosylation of high-mobility-group I (HMGI) proteins. *The Biochemical journal*, 317 (Pt 3), 865-870.

Gilchrist, M., Thorsson, V., Li, B., Rust, A.G., Korb, M., Roach, J.C., Kennedy, K., Hai, T., Bolouri, H. & Aderem, A. (2006) Systems biology approaches identify ATF3 as a negative regulator of Toll-like receptor 4. *Nature*, 441, 173-178.

Gillen, C., Jander, S. & Stoll, G. (1998) Sequential expression of mRNA for proinflammatory cytokines and interleukin-10 in the rat peripheral nervous system: comparison between immune-mediated demyelination and Wallerian degeneration. *Journal of neuroscience research*, 51, 489-496.

Goethals, S., Ydens, E., Timmerman, V. & Janssens, S. (2010) Toll-like receptor expression in the peripheral nerve. *Glia*, 58, 1701-1709.

Goldstein, M.E., House, S.B. & Gainer, H. (1991) NF-L and peripherin immunoreactivities define distinct classes of rat sensory ganglion cells. *Journal of neuroscience research*, 30, 92-104.

Golz, G., Uhlmann, L., Ludecke, D., Markgraf, N., Nitsch, R. & Hendrix, S. (2006) The cytokine/neurotrophin axis in peripheral axon outgrowth. *Eur J Neurosci*, 24, 2721-2730.

Gong, G., Yuan, L.B., Hu, L., Wu, W., Yin, L., Hou, J.L., Liu, Y.H. & Zhou, L.S. (2012) Glycyrrhizin attenuates rat ischemic spinal cord injury by suppressing inflammatory cytokines and HMGB1. *Acta pharmacologica Sinica*, 33, 11-18.

Gonzalez, M.F., Shiraishi, K., Hisanaga, K., Sagar, S.M., Mandabach, M. & Sharp, F.R. (1989) Heat shock proteins as markers of neural injury. *Brain research. Molecular brain research*, 6, 93-100.

Goodwin, G.H., Nicolas, R.H. & Johns, E.W. (1975) An improved large scale fractionation of high mobility group non-histone chromatin proteins. *Biochim Biophys Acta*, 405, 280-291.

Goodwin, G.H., Sanders, C. & Johns, E.W. (1973) A new group of chromatin-associated proteins with a high content of acidic and basic amino acids. *European journal of biochemistry / FEBS*, 38, 14-19.

Gorina, R., Font-Nieves, M., Marquez-Kisinousky, L., Santalucia, T. & Planas, A.M. (2011) Astrocyte TLR4 activation induces a proinflammatory environment through the interplay between MyD88-dependent NFkappaB signaling, MAPK, and Jak1/Stat1 pathways. *Glia*, 59, 242-255.

Govrin-Lippmann, R. & Devor, M. (1978) Ongoing activity in severed nerves: source and variation with time. *Brain Res*, 159, 406-410.

Guazzi, S., Strangio, A., Franzi, A.T. & Bianchi, M.E. (2003) HMGB1, an architectural chromatin protein and extracellular signalling factor, has a spatially and temporally restricted expression pattern in mouse brain. *Gene expression patterns : GEP*, 3, 29-33.

Handwerker, H.O., Kilo, S. & Reeh, P.W. (1991) Unresponsive afferent nerve fibres in the sural nerve of the rat. *The Journal of physiology*, 435, 229-242.

Hans, P., Bonhomme, V., Collette, J. & Moonen, G. (1993) Neuron-specific enolase as a marker of in vitro neuronal damage. Part I: Assessment of neuron-specific enolase as a quantitative and specific marker of neuronal damage. *Journal of neurosurgical anesthesiology*, 5, 111-116.

Hardman, C.H., Broadhurst, R.W., Raine, A.R., Grasser, K.D., Thomas, J.O. & Laue, E.D. (1995) Structure of the A-domain of HMG1 and its interaction with DNA as studied by heteronuclear three- and four-dimensional NMR spectroscopy. *Biochemistry*, 34, 16596-16607.

Harper, A.A. & Lawson, S.N. (1985a) Conduction velocity is related to morphological cell type in rat dorsal root ganglion neurones. *The Journal of physiology*, 359, 31-46.

Harper, A.A. & Lawson, S.N. (1985b) Electrical properties of rat dorsal root ganglion neurones with different peripheral nerve conduction velocities. *The Journal of physiology*, 359, 47-63.

Harris, H.E. & Raucci, A. (2006) Alarmin(g) news about danger: workshop on innate danger signals and HMGB1. *EMBO Rep*, 7, 774-778.

Hayakawa, K., Arai, K. & Lo, E.H. (2010a) Role of ERK map kinase and CRM1 in IL-1beta-stimulated release of HMGB1 from cortical astrocytes. *Glia*, 58, 1007-1015.

Hayakawa, K., Nakano, T., Irie, K., Higuchi, S., Fujioka, M., Orito, K., Iwasaki, K., Jin, G., Lo, E.H., Mishima, K. & Fujiwara, M. (2010b) Inhibition of reactive astrocytes with fluorocitrate retards neurovascular remodeling and recovery after focal cerebral ischemia in mice. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism*, 30, 871-882.

Haziot, A., Ferrero, E., Kontgen, F., Hijiya, N., Yamamoto, S., Silver, J., Stewart, C.L. & Goyert, S.M. (1996) Resistance to endotoxin shock and reduced dissemination of gram-negative bacteria in CD14-deficient mice. *Immunity*, 4, 407-414.

Herzberg, U., Eliav, E., Dorsey, J.M., Gracely, R.H. & Kopin, I.J. (1997) NGF involvement in pain induced by chronic constriction injury of the rat sciatic nerve. *Neuroreport*, 8, 1613-1618.

Hirsch, T., Spielmann, M., Zuhaili, B., Fossum, M., Metzig, M., Koehler, T., Steinau, H.U., Yao, F., Onderdonk, A.B., Steinstraesser, L. & Eriksson, E. (2009) Human beta-defensin-3 promotes wound healing in infected diabetic wounds. *The journal of gene medicine*, 11, 220-228.

Hoebe, K., Du, X., Georgel, P., Janssen, E., Tabeta, K., Kim, S.O., Goode, J., Lin, P., Mann, N., Mudd, S., Crozat, K., Sovath, S., Han, J. & Beutler, B. (2003) Identification of Lps2 as a key transducer of MyD88-independent TIR signalling. *Nature*, 424, 743-748.

Hofmann, H.A., De Vry, J., Siegling, A., Spreyer, P. & Denzer, D. (2003) Pharmacological sensitivity and gene expression analysis of the tibial nerve injury model of neuropathic pain. *Eur J Pharmacol*, 470, 17-25.

Hokfelt, T., Elde, R., Johansson, O., Luft, R., Nilsson, G. & Arimura, A. (1976) Immunohistochemical evidence for separate populations of somatostatin-containing and substance P-containing primary afferent neurons in the rat. *Neuroscience*, 1, 131-136.

Hoppe, G., Talcott, K.E., Bhattacharya, S.K., Crabb, J.W. & Sears, J.E. (2006) Molecular basis for the redox control of nuclear transport of the structural chromatin protein Hmgb1. *Experimental cell research*, 312, 3526-3538.

Hori, O., Brett, J., Slattery, T., Cao, R., Zhang, J., Chen, J.X., Nagashima, M., Lundh, E.R., Vijay, S., Nitecki, D. & et al. (1995) The receptor for advanced glycation end products (RAGE) is a cellular binding site for amphoterin. Mediation of neurite outgrowth and co-expression of rage and amphoterin in the developing nervous system. *The Journal of biological chemistry*, 270, 25752-25761.

Hornung, V., Rothenfusser, S., Britsch, S., Krug, A., Jahrsdorfer, B., Giese, T., Endres, S. & Hartmann, G. (2002) Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. *J Immunol*, 168, 4531-4537.

Hoshino, K., Takeuchi, O., Kawai, T., Sanjo, H., Ogawa, T., Takeda, Y., Takeda, K. & Akira, S. (1999) Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. *J Immunol*, 162, 3749-3752.

Hou, L. & Wang, X. (2001) PKC and PKA, but not PKG mediate LPS-induced CGRP release and [Ca(2+)](i) elevation in DRG neurons of neonatal rats. *Journal of neuroscience research*, 66, 592-600.

Hreggvidsdottir, H.S., Lundberg, A.M., Aveberger, A.C., Klevenvall, L., Andersson, U. & Harris, H.E. (2011) HMGB1-partner molecule complexes enhance cytokine production by signaling through the partner molecule receptor. *Molecular medicine*.

Hreggvidsdottir, H.S., Ostberg, T., Wahamaa, H., Schierbeck, H., Aveberger, A.C., Klevenvall, L., Palmblad, K., Ottosson, L., Andersson, U. & Harris, H.E. (2009) The alarmin HMGB1 acts in synergy with endogenous and exogenous danger signals to promote inflammation. *Journal of leukocyte biology*, 86, 655-662.

Hua, F., Ma, J., Ha, T., Xia, Y., Kelley, J., Williams, D.L., Kao, R.L., Browder, I.W., Schweitzer, J.B., Kalbfleisch, J.H. & Li, C. (2007) Activation of Toll-like receptor 4 signaling contributes to hippocampal neuronal death following global cerebral ischemia/reperfusion. *J Neuroimmunol*, 190, 101-111.

Huang, W., Tang, Y. & Li, L. (2010) HMGB1, a potent proinflammatory cytokine in sepsis. *Cytokine*, 51, 119-126.

Hunt, D., Raivich, G. & Anderson, P.N. (2012) Activating transcription factor 3 and the nervous system. *Frontiers in molecular neuroscience*, 5, 7.

Hunt, S.P. & Rossi, J. (1985) Peptide- and non-peptide-containing unmyelinated primary afferents: the parallel processing of nociceptive information. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, 308, 283-289.

Husebye, H., Halaas, O., Stenmark, H., Tunheim, G., Sandanger, O., Bogen, B., Brech, A., Latz, E. & Espevik, T. (2006) Endocytic pathways regulate Toll-like receptor 4 signaling and link innate and adaptive immunity. *EMBO J*, 25, 683-692.

Hutchinson, M.R., Lewis, S.S., Coats, B.D., Rezvani, N., Zhang, Y., Wieseler, J.L., Somogyi, A.A., Yin, H., Maier, S.F., Rice, K.C. & Watkins, L.R. (2010a) Possible involvement of toll-like receptor 4/myeloid differentiation factor-2 activity of opioid inactive isomers causes spinal proinflammation and related behavioral consequences. *Neuroscience*, 167, 880-893.

Hutchinson, M.R., Ramos, K.M., Loram, L.C., Wieseler, J., Sholar, P.W., Kearney, J.J., Lewis, M.T., Crysdale, N.Y., Zhang, Y., Harrison, J.A., Maier, S.F., Rice, K.C. & Watkins, L.R. (2009) Evidence for a role of heat shock protein-90 in toll like receptor 4 mediated pain enhancement in rats. *Neuroscience*, 164, 1821-1832.

Hutchinson, M.R., Zhang, Y., Brown, K., Coats, B.D., Shridhar, M., Sholar, P.W., Patel, S.J., Crysdale, N.Y., Harrison, J.A., Maier, S.F., Rice, K.C. & Watkins, L.R. (2008) Non-stereoselective reversal of neuropathic pain by naloxone and naltrexone: involvement of toll-like receptor 4 (TLR4). *Eur J Neurosci*, 28, 20-29.

Hutchinson, M.R., Zhang, Y., Shridhar, M., Evans, J.H., Buchanan, M.M., Zhao, T.X., Slivka, P.F., Coats, B.D., Rezvani, N., Wieseler, J., Hughes, T.S., Landgraf, K.E., Chan, S., Fong, S., Phipps, S., Falke, J.J., Leinwand, L.A., Maier, S.F., Yin, H., Rice, K.C. & Watkins, L.R. (2010b) Evidence that opioids may have toll-like receptor 4 and MD-2 effects. *Brain Behav Immun*, 24, 83-95.

Huttunen, H.J., Fages, C., Kuja-Panula, J., Ridley, A.J. & Rauvala, H. (2002) Receptor for advanced glycation end products-binding COOH-terminal motif of amphoterin inhibits invasive migration and metastasis. *Cancer research*, 62, 4805-4811.

Huttunen, H.J., Kuja-Panula, J., Sorci, G., Agneletti, A.L., Donato, R. & Rauvala, H. (2000) Coregulation of neurite outgrowth and cell survival by amphoterin and S100 proteins through receptor for advanced glycation end products (RAGE) activation. *J Biol Chem*, 275, 40096-40105.

Huttunen, H.J. & Rauvala, H. (2004) Amphoterin as an extracellular regulator of cell motility: from discovery to disease. *J Intern Med*, 255, 351-366.

Inbal, R., Rousso, M., Ashur, H., Wall, P.D. & Devor, M. (1987) Collateral sprouting in skin and sensory recovery after nerve injury in man. *Pain*, 28, 141-154.

Ito, I., Fukazawa, J. & Yoshida, M. (2007) Post-translational methylation of high mobility group box 1 (HMGB1) causes its cytoplasmic localization in neutrophils. *J Biol Chem*, 282, 16336-16344.

Ivanov, S., Dragoi, A.M., Wang, X., Dallacosta, C., Louten, J., Musco, G., Sitia, G., Yap, G.S., Wan, Y., Biron, C.A., Bianchi, M.E., Wang, H. & Chu, W.M. (2007) A novel role for HMGB1 in TLR9-mediated inflammatory responses to CpG-DNA. *Blood*, 110, 1970-1981.

Jahnel, R., Bender, O., Munter, L.M., Dreger, M., Gillen, C. & Hucho, F. (2003) Dual expression of mouse and rat VRL-1 in the dorsal root ganglion derived cell line F-11 and biochemical analysis of VRL-1 after heterologous expression. *European journal of biochemistry / FEBS*, 270, 4264-4271.

Jang, J.H., Nam, T.S., Paik, K.S. & Leem, J.W. (2004) Involvement of peripherally released substance P and calcitonin gene-related peptide in mediating mechanical hyperalgesia in a traumatic neuropathy model of the rat. *Neurosci Lett*, 360, 129-132.

Jankowski, M.P., Cornuet, P.K., McIlwrath, S., Koerber, H.R. & Albers, K.M. (2006) SRY-box containing gene 11 (Sox11) transcription factor is required for neuron survival and neurite growth. *Neuroscience*, 143, 501-514.

Jessell, T.M. & Dodd, J. (1985) Structure and expression of differentiation antigens on functional subclasses of primary sensory neurons. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, 308, 271-281.

Ji, R.R., Samad, T.A., Jin, S.X., Schmoll, R. & Woolf, C.J. (2002) p38 MAPK activation by NGF in primary sensory neurons after inflammation increases TRPV1 levels and maintains heat hyperalgesia. *Neuron*, 36, 57-68.

Jiang, Z., Georgel, P., Du, X., Shamel, L., Sovath, S., Mudd, S., Huber, M., Kalis, C., Keck, S., Galanos, C., Freudenberg, M. & Beutler, B. (2005) CD14 is required for MyD88-independent LPS signaling. *Nat Immunol*, 6, 565-570.

Jin, X. & Gereau, R.W.t. (2006) Acute p38-mediated modulation of tetrodotoxin-resistant sodium channels in mouse sensory neurons by tumor necrosis factor-alpha. *J Neurosci*, 26, 246-255.

Jo, H.Y., Kim, S.Y., Lee, S., Jeong, S., Kim, S.J., Kang, T.M. & Lee, K.Y. (2011) Kir3.1 channel is functionally involved in TLR4-mediated signaling. *Biochem Biophys Res Commun*, 407, 687-691.

Ju, B.G., Lunyak, V.V., Perissi, V., Garcia-Bassets, I., Rose, D.W., Glass, C.K. & Rosenfeld, M.G. (2006) A topoisomerase IIbeta-mediated dsDNA break required for regulated transcription. *Science*, 312, 1798-1802.

Ju, G., Hokfelt, T., Brodin, E., Fahrenkrug, J., Fischer, J.A., Frey, P., Elde, R.P. & Brown, J.C. (1987) Primary sensory neurons of the rat showing calcitonin gene-related peptide immunoreactivity and their relation to substance P-, somatostatin-, galanin-, vasoactive intestinal polypeptide- and cholecystokinin-immunoreactive ganglion cells. *Cell Tissue Res*, 247, 417-431.

Jung, H., Bhangoo, S., Banisadr, G., Freitag, C., Ren, D., White, F.A. & Miller, R.J. (2009) Visualization of chemokine receptor activation in transgenic mice reveals peripheral activation of CCR2 receptors in states of neuropathic pain. *J Neurosci*, 29, 8051-8062.

Jung, H., Toth, P.T., White, F.A. & Miller, R.J. (2008) Monocyte chemoattractant protein-1 functions as a neuromodulator in dorsal root ganglia neurons. *J Neurochem*, 104, 254-263.

Kagan, J.C., Su, T., Horng, T., Chow, A., Akira, S. & Medzhitov, R. (2008) TRAM couples endocytosis of Toll-like receptor 4 to the induction of interferon-beta. *Nat Immunol*, 9, 361-368.

Kajander, K.C. & Bennett, G.J. (1992) Onset of a painful peripheral neuropathy in rat: a partial and differential deafferentation and spontaneous discharge in A beta and A delta primary afferent neurons. *J Neurophysiol*, 68, 734-744.

Kajander, K.C., Wakisaka, S. & Bennett, G.J. (1992) Spontaneous discharge originates in the dorsal root ganglion at the onset of a painful peripheral neuropathy in the rat. *Neurosci Lett*, 138, 225-228.

Kapadia, S.E. & LaMotte, C.C. (1987) Deafferentation-induced alterations in the rat dorsal horn: I. Comparison of peripheral nerve injury vs. rhizotomy effects on presynaptic, postsynaptic, and glial processes. *The Journal of comparative neurology*, 266, 183-197.

Kashiba, H., Ueda, Y. & Senba, E. (1996) Coexpression of preprotachykinin-A, alpha-calcitonin gene-related peptide, somatostatin, and neurotrophin receptor family messenger RNAs in rat dorsal root ganglion neurons. *Neuroscience*, 70, 179-189.

Kawabata, H., Setoguchi, T., Yone, K., Souda, M., Yoshida, H., Kawahara, K., Maruyama, I. & Komiya, S. (2010) High mobility group box 1 is upregulated after spinal cord injury and is associated with neuronal cell apoptosis. *Spine (Phila Pa 1976)*, 35, 1109-1115.

Kawagoe, T., Sato, S., Matsushita, K., Kato, H., Matsui, K., Kumagai, Y., Saitoh, T., Kawai, T., Takeuchi, O. & Akira, S. (2008) Sequential control of Toll-like receptor-dependent responses by IRAK1 and IRAK2. *Nat Immunol*, 9, 684-691.

Kawai, T., Adachi, O., Ogawa, T., Takeda, K. & Akira, S. (1999) Unresponsiveness of MyD88-deficient mice to endotoxin. *Immunity*, 11, 115-122.

Kawai, T. & Akira, S. (2010) The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat Immunol*, 11, 373-384.

Kawai, T., Takeuchi, O., Fujita, T., Inoue, J., Muhlradt, P.F., Sato, S., Hoshino, K. & Akira, S. (2001) Lipopolysaccharide stimulates the MyD88-independent pathway and results in activation of IFN-regulatory factor 3 and the expression of a subset of lipopolysaccharide-inducible genes. *J Immunol*, 167, 5887-5894.

Kessler, W., Kirchhoff, C., Reeh, P.W. & Handwerker, H.O. (1992) Excitation of cutaneous afferent nerve endings in vitro by a combination of inflammatory mediators and conditioning effect of substance P. *Experimental brain research. Experimentelle Hirnforschung. Experimentation cerebrale*, 91, 467-476.

Ketz, A.K. (2008) The experience of phantom limb pain in patients with combat-related traumatic amputations. *Archives of physical medicine and rehabilitation*, 89, 1127-1132.

Kikuchi, K., Uchikado, H., Miura, N., Morimoto, Y., Ito, T., Tancharoen, S., Miyata, K., Sakamoto, R., Kikuchi, C., Iida, N., Shiomi, N., Kuramoto, T., Miyagi, N. & Kawahara, K.I. (2011) HMGB1 as a therapeutic target in spinal cord injury: A hypothesis for novel therapy development. *Experimental and therapeutic medicine*, 2, 767-770.

Kim, D.S., Choi, J.O., Rim, H.D. & Cho, H.J. (2002) Downregulation of voltage-gated potassium channel alpha gene expression in dorsal root ganglia following chronic constriction injury of the rat sciatic nerve. *Brain research. Molecular brain research*, 105, 146-152.

Kim, H.M., Park, B.S., Kim, J.I., Kim, S.E., Lee, J., Oh, S.C., Enkhbayar, P., Matsushima, N., Lee, H., Yoo, O.J. & Lee, J.O. (2007a) Crystal structure of the TLR4-MD-2 complex with bound endotoxin antagonist Eritoran. *Cell*, 130, 906-917.

Kim, J.B., Lim, C.M., Yu, Y.M. & Lee, J.K. (2008) Induction and subcellular localization of high-mobility group box-1 (HMGB1) in the postischemic rat brain. *Journal of neuroscience research*, 86, 1125-1131.

Kim, J.B., Sig Choi, J., Yu, Y.M., Nam, K., Piao, C.S., Kim, S.W., Lee, M.H., Han, P.L., Park, J.S. & Lee, J.K. (2006) HMGB1, a novel cytokine-like mediator linking acute neuronal death and delayed neuroinflammation in the postischemic brain. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 26, 6413-6421.

Kim, S.H. & Chung, J.M. (1992) An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. *Pain*, 50, 355-363.

Kim, S.W., Jin, Y., Shin, J.H., Kim, I.D., Lee, H.K., Park, S., Han, P.L. & Lee, J.K. (2012) Glycyrrhizic acid affords robust neuroprotection in the postischemic brain via anti-inflammatory effect by inhibiting HMGB1 phosphorylation and secretion. *Neurobiol Dis*, 46, 147-156.

Kim, S.W., Lim, C.M., Kim, J.B., Shin, J.H., Lee, S., Lee, M. & Lee, J.K. (2011) Extracellular HMGB1 released by NMDA treatment confers neuronal apoptosis via RAGE-p38 MAPK/ERK signaling pathway. *Neurotox Res*, 20, 159-169.

Kim, T.W., Staschke, K., Bulek, K., Yao, J., Peters, K., Oh, K.H., Vandenburg, Y., Xiao, H., Qian, W., Hamilton, T., Min, B., Sen, G., Gilmour, R. & Li, X. (2007b) A critical role for IRAK4 kinase activity in Toll-like receptor-mediated innate immunity. *J Exp Med*, 204, 1025-1036.

Kinnman, E. & Aldskogius, H. (1986) Collateral sprouting of sensory axons in the glabrous skin of the hindpaw after chronic sciatic nerve lesion in adult and neonatal rats: a morphological study. *Brain Res*, 377, 73-82.

Kishore, S.P., Bungum, M.K., Platt, J.L. & Brunn, G.J. (2005) Selective suppression of Toll-like receptor 4 activation by chemokine receptor 4. *FEBS letters*, 579, 699-704.

Klune, J.R., Dhupar, R., Cardinal, J., Billiar, T.R. & Tsung, A. (2008) HMGB1: endogenous danger signaling. *Molecular medicine*, 14, 476-484.

Knyihar-Csillik, E. & Csillik, B. (1981) FRAP: histochemistry of the primary nociceptive neuron. *Progress in histochemistry and cytochemistry*, 14, 1-137.

Kohno, T., Moore, K.A., Baba, H. & Woolf, C.J. (2003) Peripheral nerve injury alters excitatory synaptic transmission in lamina II of the rat dorsal horn. *The Journal of physiology*, 548, 131-138.

Kovalsky, Y., Amir, R. & Devor, M. (2009) Simulation in sensory neurons reveals a key role for delayed Na+ current in subthreshold oscillations and ectopic discharge: implications for neuropathic pain. *J Neurophysiol*, 102, 1430-1442.

Kruger, L., Perl, E.R. & Sedivec, M.J. (1981) Fine structure of myelinated mechanical nociceptor endings in cat hairy skin. *The Journal of comparative neurology*, 198, 137-154.

Kuang, X., Huang, Y., Gu, H.F., Zu, X.Y., Zou, W.Y., Song, Z.B. & Guo, Q.L. (2012) Effects of intrathecal epigallocatechin gallate, an inhibitor of Toll-like receptor 4, on chronic neuropathic pain in rats. *Eur J Pharmacol*, 676, 51-56.

Kurejova, M., Nattenmuller, U., Hildebrandt, U., Selvaraj, D., Stosser, S. & Kuner, R. (2010) An improved behavioural assay demonstrates that ultrasound vocalizations constitute a reliable indicator of chronic cancer pain and neuropathic pain. *Mol Pain*, 6, 18.

Kurt-Jones, E.A., Popova, L., Kwinn, L., Haynes, L.M., Jones, L.P., Tripp, R.A., Walsh, E.E., Freeman, M.W., Golenbock, D.T., Anderson, L.J. & Finberg, R.W. (2000) Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus. *Nat Immunol*, 1, 398-401.

Kwon, J., Imbalzano, A.N., Matthews, A. & Oettinger, M.A. (1998) Accessibility of nucleosomal DNA to V(D)J cleavage is modulated by RSS positioning and HMG1. *Mol Cell*, 2, 829-839.

LaBuda, C.J. & Little, P.J. (2005) Pharmacological evaluation of the selective spinal nerve ligation model of neuropathic pain in the rat. *Journal of neuroscience methods*, 144, 175-181.

Lacroix-Fralish, M.L. & Mogil, J.S. (2009) Progress in genetic studies of pain and analgesia. *Annual review of pharmacology and toxicology*, 49, 97-121.

Lamkanfi, M., Sarkar, A., Vande Walle, L., Vitari, A.C., Amer, A.O., Wewers, M.D., Tracey, K.J., Kanneganti, T.D. & Dixit, V.M. (2010) Inflammasome-dependent release of the alarmin HMGB1 in endotoxemia. *J Immunol*, 185, 4385-4392.

Langford, D.J., Bailey, A.L., Chanda, M.L., Clarke, S.E., Drummond, T.E., Echols, S., Glick, S., Ingrao, J., Klassen-Ross, T., Lacroix-Fralish, M.L., Matsumiya, L., Sorge, R.E., Sotocinal, S.G., Tabaka, J.M., Wong, D., van den Maagdenberg, A.M., Ferrari, M.D., Craig, K.D. & Mogil, J.S. (2010) Coding of facial expressions of pain in the laboratory mouse. *Nature methods*, 7, 447-449.

Latremoliere, A. & Woolf, C.J. (2009) Central sensitization: a generator of pain hypersensitivity by central neural plasticity. *J Pain*, 10, 895-926.

Latz, E., Visintin, A., Lien, E., Fitzgerald, K.A., Monks, B.G., Kurt-Jones, E.A., Golenbock, D.T. & Espevik, T. (2002) Lipopolysaccharide rapidly traffics to and from the Golgi apparatus with the toll-like receptor 4-MD-2-CD14 complex in a process that is distinct from the initiation of signal transduction. *J Biol Chem*, 277, 47834-47843.

Lawson, J.J., McIlwrath, S.L., Woodbury, C.J., Davis, B.M. & Koerber, H.R. (2008) TRPV1 unlike TRPV2 is restricted to a subset of mechanically insensitive cutaneous nociceptors responding to heat. *J Pain*, 9, 298-308.

Lawson, S.N. (2002) Phenotype and function of somatic primary afferent nociceptive neurones with C-, Adelta- or Aalpha/beta-fibres. *Experimental physiology*, 87, 239-244.

Lawson, S.N., Caddy, K.W. & Biscoe, T.J. (1974) Development of rat dorsal root ganglion neurones. Studies of cell birthdays and changes in mean cell diameter. *Cell Tissue Res*, 153, 399-413.

Lawson, S.N., Crepps, B.A. & Perl, E.R. (1997) Relationship of substance P to afferent characteristics of dorsal root ganglion neurones in guinea-pig. *The Journal of physiology*, 505 (Pt 1), 177-191.

Lawson, S.N., Harper, A.A., Harper, E.I., Garson, J.A. & Anderton, B.H. (1984) A monoclonal antibody against neurofilament protein specifically labels a subpopulation of rat sensory neurones. *The Journal of comparative neurology*, 228, 263-272.

Le Bars, D., Gozariu, M. & Cadden, S.W. (2001) Animal models of nociception. *Pharmacological reviews*, 53, 597-652.

Lee, B.H., Won, R., Baik, E.J., Lee, S.H. & Moon, C.H. (2000) An animal model of neuropathic pain employing injury to the sciatic nerve branches. *Neuroreport*, 11, 657-661.

Lee, H., Park, C., Cho, I.H., Kim, H.Y., Jo, E.K., Lee, S., Kho, H.S., Choi, S.Y., Oh, S.B., Park, K., Kim, J.S. & Lee, S.J. (2007) Double-stranded RNA induces iNOS gene expression in Schwann cells, sensory neuronal death, and peripheral nerve demyelination. *Glia*, 55, 712-722.

Lee, H., Shin, N., Song, M., Kang, U.B., Yeom, J., Lee, C., Ahn, Y.H., Yoo, J.S., Paik, Y.K. & Kim, H. (2010) Analysis of nuclear high mobility group box 1 (HMGB1)-binding proteins in colon cancer cells: clustering with proteins involved in secretion and extranuclear function. *J Proteome Res*, 9, 4661-4670.

Leem, J.W., Willis, W.D. & Chung, J.M. (1993) Cutaneous sensory receptors in the rat foot. *J Neurophysiol*, 69, 1684-1699.

Lehnardt, S., Lachance, C., Patrizi, S., Lefebvre, S., Follett, P.L., Jensen, F.E., Rosenberg, P.A., Volpe, J.J. & Vartanian, T. (2002) The toll-like receptor TLR4 is necessary for lipopolysaccharide-induced oligodendrocyte injury in the CNS. *J Neurosci*, 22, 2478-2486.

Lehnardt, S., Massillon, L., Follett, P., Jensen, F.E., Ratan, R., Rosenberg, P.A., Volpe, J.J. & Vartanian, T. (2003) Activation of innate immunity in the CNS triggers neurodegeneration through a Toll-like receptor 4-dependent pathway. *Proc Natl Acad Sci U S A*, 100, 8514-8519.

Lehnardt, S., Schott, E., Trimbuch, T., Laubisch, D., Krueger, C., Wulczyn, G., Nitsch, R. & Weber, J.R. (2008) A vicious cycle involving release of heat shock protein 60 from injured cells and activation of toll-like receptor 4 mediates neurodegeneration in the CNS. *J Neurosci*, 28, 2320-2331.

Leitner, M.L., Molliver, D.C., Osborne, P.A., Vejsada, R., Golden, J.P., Lampe, P.A., Kato, A.C., Milbrandt, J. & Johnson, E.M., Jr. (1999) Analysis of the retrograde transport of glial cell line-derived neurotrophic factor (GDNF), neurturin, and persephin suggests that in vivo signaling for the GDNF family is GFRalpha coreceptor-specific. *J Neurosci*, 19, 9322-9331.

Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J.M. & Hoffmann, J.A. (1996) The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. *Cell*, 86, 973-983.

Lewis, S.S., Hutchinson, M.R., Rezvani, N., Loram, L.C., Zhang, Y., Maier, S.F., Rice, K.C. & Watkins, L.R. (2010) Evidence that intrathecal morphine-3-glucuronide may cause pain enhancement via toll-like receptor 4/MD-2 and interleukin-1beta. *Neuroscience*, 165, 569-583.

Li, J., Kokkola, R., Tabibzadeh, S., Yang, R., Ochani, M., Qiang, X., Harris, H.E., Czura, C.J., Wang, H., Ulloa, L., Wang, H., Warren, H.S., Moldawer, L.L., Fink, M.P., Andersson, U., Tracey, K.J. & Yang, H. (2003) Structural basis for the proinflammatory cytokine activity of high mobility group box 1. *Mol Med*, 9, 37-45.

Li, T., Hu, J. & Li, L. (2004) Characterization of Tollip protein upon Lipopolysaccharide challenge. *Molecular immunology*, 41, 85-92.

Light, A.R. & Perl, E.R. (1979) Spinal termination of functionally identified primary afferent neurons with slowly conducting myelinated fibers. *The Journal of comparative neurology*, 186, 133-150.

Light, A.R. & Perl, E.R. (2003) Unmyelinated afferent fibers are not only for pain anymore. *The Journal of comparative neurology*, 461, 137-139.

Lindwall, C. & Kanje, M. (2005) Retrograde axonal transport of JNK signaling molecules influence injury induced nuclear changes in p-c-Jun and ATF3 in adult rat sensory neurons. *Mol Cell Neurosci*, 29, 269-282.

Lirk, P., Poroli, M., Rigaud, M., Fuchs, A., Fillip, P., Huang, C.Y., Ljubkovic, M., Sapunar, D. & Hogan, Q. (2008) Modulators of calcium influx regulate membrane excitability in rat dorsal root ganglion neurons. *Anesth Analg*, 107, 673-685.

Litvak, V., Ramsey, S.A., Rust, A.G., Zak, D.E., Kennedy, K.A., Lampano, A.E., Nykter, M., Shmulevich, I. & Aderem, A. (2009) Function of C/EBPdelta in a regulatory circuit that discriminates between transient and persistent TLR4-induced signals. *Nat Immunol*, 10, 437-443.

Liu, H., Wang, H., Sheng, M., Jan, L.Y., Jan, Y.N. & Basbaum, A.I. (1994) Evidence for presynaptic N-methyl-D-aspartate autoreceptors in the spinal cord dorsal horn. *Proc Natl Acad Sci U S A*, 91, 8383-8387.

Liu, H., Yao, Y.M., Ding, L.H., Zhang, H., Yuan, B., Song, Q., Ye, Q.N., Huang, C.F. & Sheng, Z.Y. (2009) High mobility group box-1 protein acts as a coactivator of nuclear factor of activated T cells-2 in promoting interleukin-2 transcription. *The international journal of biochemistry & cell biology*, 41, 641-648.

Liu, J., Liu, Y., Zhang, H., Chen, G., Wang, K. & Xiao, X. (2008) KLF4 promotes the expression, translocation, and releas eof HMGB1 in RAW264.7 macrophages in response to LPS. *Shock*, 30, 260-266.

Liu, K., Mori, S., Takahashi, H.K., Tomono, Y., Wake, H., Kanke, T., Sato, Y., Hiraga, N., Adachi, N., Yoshino, T. & Nishibori, M. (2007) Anti-high mobility group box 1 monoclonal antibody ameliorates brain infarction induced by transient ischemia in rats. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, 21, 3904-3916.

Liu, S., Stolz, D.B., Sappington, P.L., Macias, C.A., Killeen, M.E., Tenhunen, J.J., Delude, R.L. & Fink, M.P. (2006) HMGB1 is secreted by immunostimulated enterocytes and contributes to cytomix-induced hyperpermeability of Caco-2 monolayers. *Am J Physiol Cell Physiol*, 290, C990-999.

Liu, X., Eschenfelder, S., Blenk, K.H., Janig, W. & Habler, H. (2000) Spontaneous activity of axotomized afferent neurons after L5 spinal nerve injury in rats. *Pain*, 84, 309-318.

Lo Coco, D., Veglianese, P., Allievi, E. & Bendotti, C. (2007) Distribution and cellular localization of high mobility group box protein 1 (HMGB1) in the spinal cord of a transgenic mouse model of ALS. *Neurosci Lett*, 412, 73-77.

Loeser, J.D. & Treede, R.D. (2008) The Kyoto protocol of IASP Basic Pain Terminology. *Pain*, 137, 473-477.

Lopes, P. & Couture, R. (1997) Localization of bradykinin-like immunoreactivity in the rat spinal cord: effects of capsaicin, melittin, dorsal rhizotomy and peripheral axotomy. *Neuroscience*, 78, 481-497.

Lorenzo, L.E., Ramien, M., St Louis, M., De Koninck, Y. & Ribeiro-da-Silva, A. (2008) Postnatal changes in the Rexed lamination and markers of nociceptive afferents in the superficial dorsal horn of the rat. *The Journal of comparative neurology*, 508, 592-604.

Lotze, M.T. & Tracey, K.J. (2005) High-mobility group box 1 protein (HMGB1): nuclear weapon in the immune arsenal. *Nat Rev Immunol*, 5, 331-342.

Lu, Y.C., Yeh, W.C. & Ohashi, P.S. (2008) LPS/TLR4 signal transduction pathway. *Cytokine*, 42, 145-151.

Lum, H.K. & Lee, K.L. (2001) The human HMGB1 promoter is modulated by a silencer and an enhancer-containing intron. *Biochim Biophys Acta*, 1520, 79-84.

Lumpkin, E.A. & Caterina, M.J. (2007) Mechanisms of sensory transduction in the skin. *Nature*, 445, 858-865.

Luo, Z.D., Chaplan, S.R., Higuera, E.S., Sorkin, L.S., Stauderman, K.A., Williams, M.E. & Yaksh, T.L. (2001) Upregulation of dorsal root ganglion (alpha)2(delta) calcium channel subunit and its correlation with allodynia in spinal nerve-injured rats. *J Neurosci*, 21, 1868-1875.

Lye, E., Dhanji, S., Calzascia, T., Elford, A.R. & Ohashi, P.S. (2008) IRAK-4 kinase activity is required for IRAK-4-dependent innate and adaptive immune responses. *European journal of immunology*, 38, 870-876.

Lye, E., Mirtsos, C., Suzuki, N., Suzuki, S. & Yeh, W.C. (2004) The role of interleukin 1 receptor-associated kinase-4 (IRAK-4) kinase activity in IRAK-4-mediated signaling. *J Biol Chem*, 279, 40653-40658.

Lynn, B. & Carpenter, S.E. (1982) Primary afferent units from the hairy skin of the rat hind limb. *Brain Res*, 238, 29-43.

Ma, C. & LaMotte, R.H. (2005) Enhanced excitability of dissociated primary sensory neurons after chronic compression of the dorsal root ganglion in the rat. *Pain*, 113, 106-112.

Ma, C., Shu, Y., Zheng, Z., Chen, Y., Yao, H., Greenquist, K.W., White, F.A. & LaMotte, R.H. (2003) Similar electrophysiological changes in axotomized and neighboring intact dorsal root ganglion neurons. *J Neurophysiol*, 89, 1588-1602.

Ma, Q.P. & Hargreaves, R.J. (2000) Localization of N-methyl-D-aspartate NR2B subunits on primary sensory neurons that give rise to small-caliber sciatic nerve fibers in rats. *Neuroscience*, 101, 699-707.

Mabuchi, A., Wake, K., Marlini, M., Watanabe, H. & Wheatley, A.M. (2009) Protection by glycyrrhizin against warm ischemia-reperfusion-induced cellular injury and derangement of the microcirculatory blood flow in the rat liver. *Microcirculation*, 16, 364-376.

MacIver, M.B. & Tanelian, D.L. (1993a) Free nerve ending terminal morphology is fiber type specific for A delta and C fibers innervating rabbit corneal epithelium. *J Neurophysiol*, 69, 1779-1783.

MacIver, M.B. & Tanelian, D.L. (1993b) Structural and functional specialization of A delta and C fiber free nerve endings innervating rabbit corneal epithelium. *J Neurosci*, 13, 4511-4524.

Mack, T.G., Reiner, M., Beirowski, B., Mi, W., Emanuelli, M., Wagner, D., Thomson, D., Gillingwater, T., Court, F., Conforti, L., Fernando, F.S., Tarlton, A., Andressen, C., Addicks, K., Magni, G., Ribchester, R.R., Perry, V.H. & Coleman, M.P. (2001) Wallerian degeneration of injured axons and synapses is delayed by a Ube4b/Nmnat chimeric gene. *Nat Neurosci*, 4, 1199-1206.

Maguire, M.F., Ravenscroft, A., Beggs, D. & Duffy, J.P. (2006) A questionnaire study investigating the prevalence of the neuropathic component of chronic pain after thoracic surgery. *European journal of cardio-thoracic surgery : official journal of the European Association for Cardio-thoracic Surgery*, 29, 800-805.

Malin, S.A., Molliver, D.C., Koerber, H.R., Cornuet, P., Frye, R., Albers, K.M. & Davis, B.M. (2006) Glial cell line-derived neurotrophic factor family members sensitize nociceptors in vitro and produce thermal hyperalgesia in vivo. *J Neurosci*, 26, 8588-8599.

Marchand, F., Perretti, M. & McMahon, S.B. (2005) Role of the immune system in chronic pain. *Nature reviews. Neuroscience*, 6, 521-532.

Marchand, J.E., Wurm, W.H., Kato, T. & Kream, R.M. (1994) Altered tachykinin expression by dorsal root ganglion neurons in a rat model of neuropathic pain. *Pain*, 58, 219-231.

Maroso, M., Balosso, S., Ravizza, T., Liu, J., Aronica, E., Iyer, A.M., Rossetti, C., Molteni, M., Casalgrandi, M., Manfredi, A.A., Bianchi, M.E. & Vezzani, A. (2010) Toll-like receptor 4 and high-mobility group box-1 are involved in ictogenesis and can be targeted to reduce seizures. *Nat Med*, 16, 413-419.

Marubuchi, S., Okuda, T., Tagawa, K., Enokido, Y., Horiuchi, D., Shimokawa, R., Tamura, T., Qi, M.L., Eishi, Y., Watabe, K., Shibata, M., Nakagawa, M. & Okazawa, H. (2006) Hepatoma-derived growth factor, a new trophic factor for motor neurons, is up-regulated in the spinal cord of PQBP-1 transgenic mice before onset of degeneration. *J Neurochem*, 99, 70-83.

Maves, T.J., Pechman, P.S., Gebhart, G.F. & Meller, S.T. (1993) Possible chemical contribution from chromic gut sutures produces disorders of pain sensation like those seen in man. *Pain*, 54, 57-69.

Mazarati, A., Maroso, M., Iori, V., Vezzani, A. & Carli, M. (2011) High-mobility group box-1 impairs memory in mice through both toll-like receptor 4 and Receptor for Advanced Glycation End Products. *Experimental neurology*, 232, 143-148.

McGraw, J., Gaudet, A.D., Oschipok, L.W., Steeves, J.D., Poirier, F., Tetzlaff, W. & Ramer, M.S. (2005) Altered primary afferent anatomy and reduced thermal sensitivity in mice lacking galectin-1. *Pain*, 114, 7-18.

McGraw, J., McPhail, L.T., Oschipok, L.W., Horie, H., Poirier, F., Steeves, J.D., Ramer, M.S. & Tetzlaff, W. (2004) Galectin-1 in regenerating motoneurons. *Eur J Neurosci*, 20, 2872-2880.

McKemy, D.D., Neuhausser, W.M. & Julius, D. (2002) Identification of a cold receptor reveals a general role for TRP channels in thermosensation. *Nature*, 416, 52-58.

McKinney, K. & Prives, C. (2002) Efficient specific DNA binding by p53 requires both its central and C-terminal domains as revealed by studies with high-mobility group 1 protein. *Mol Cell Biol*, 22, 6797-6808.

McMahon, S., Bennett, D.L. & Bevan, S. (2006) *P.D Wall, R. Melzack (Eds.),Textbook of Pain*. Elsevier, Churchill Liningstone, New York, NY.

Medvedev, A.E., Piao, W., Shoenfelt, J., Rhee, S.H., Chen, H., Basu, S., Wahl, L.M., Fenton, M.J. & Vogel, S.N. (2007) Role of TLR4 tyrosine phosphorylation in signal transduction and endotoxin tolerance. *J Biol Chem*, 282, 16042-16053.

Medzhitov, R., Preston-Hurlburt, P. & Janeway, C.A., Jr. (1997) A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature*, 388, 394-397.

Mendell, L.M. & Wall, P.D. (1965) Responses of Single Dorsal Cord Cells to Peripheral Cutaneous Unmyelinated Fibres. *Nature*, 206, 97-99.

Mercadante, S. & Arcuri, E. (1998) Breakthrough pain in cancer patients: pathophysiology and treatment. *Cancer treatment reviews*, 24, 425-432.

Merenmies, J., Pihlaskari, R., Laitinen, J., Wartiovaara, J. & Rauvala, H. (1991) 30-kDa heparin-binding protein of brain (amphoterin) involved in neurite outgrowth. Amino acid sequence and localization in the filopodia of the advancing plasma membrane. *J Biol Chem*, 266, 16722-16729.

Merskey, H.a.B., N (1994) *A Current List with Definitions and Notes on Usage. In: Classification of Chronic Pain Descriptions of Chronic Pain Syndromes and Definitions of Pain Terms*. IASP Press, Seattle.

Meyer, R.A. & Campbell, J.N. (1981) Myelinated nociceptive afferents account for the hyperalgesia that follows a burn to the hand. *Science*, 213, 1527-1529.

Millan, M.J. (2002) Descending control of pain. *Prog Neurobiol*, 66, 355-474.

Mitola, S., Belleri, M., Urbinati, C., Coltrini, D., Sparatore, B., Pedrazzi, M., Melloni, E. & Presta, M. (2006) Cutting edge: extracellular high mobility group box-1 protein is a proangiogenic cytokine. *J Immunol*, 176, 12-15.

Moalem, G. & Tracey, D.J. (2006) Immune and inflammatory mechanisms in neuropathic pain. *Brain research reviews*, 51, 240-264.

Mollica, L., De Marchis, F., Spitaleri, A., Dallacosta, C., Pennacchini, D., Zamai, M., Agresti, A., Trisciuoglio, L., Musco, G. & Bianchi, M.E. (2007) Glycyrrhizin binds to high-mobility group box 1 protein and inhibits its cytokine activities. *Chem Biol*, 14, 431-441.

Molliver, D.C., Radeke, M.J., Feinstein, S.C. & Snider, W.D. (1995) Presence or absence of TrkA protein distinguishes subsets of small sensory neurons with unique cytochemical characteristics and dorsal horn projections. *The Journal of comparative neurology*, 361, 404-416.

Molliver, D.C., Wright, D.E., Leitner, M.L., Parsadanian, A.S., Doster, K., Wen, D., Yan, Q. & Snider, W.D. (1997) IB4-binding DRG neurons switch from NGF to GDNF dependence in early postnatal life. *Neuron*, 19, 849-861.

Morris, C.E. & Juranka, P.F. (2007) Nav channel mechanosensitivity: activation and inactivation accelerate reversibly with stretch. *Biophysical journal*, 93, 822-833.

Mouri, F., Tsukada, J., Mizobe, T., Higashi, T., Yoshida, Y., Minami, Y., Izumi, H., Kominato, Y., Kohno, K. & Tanaka, Y. (2008) Intracellular HMGB1 transactivates the human IL1B gene promoter through association with an Ets transcription factor PU.1. *European journal of haematology*, 80, 10-19.

Mueller, M., Wacker, K., Ringelstein, E.B., Hickey, W.F., Imai, Y. & Kiefer, R. (2001) Rapid response of identified resident endoneurial macrophages to nerve injury. *Am J Pathol*, 159, 2187-2197.

Muhammad, S., Barakat, W., Stoyanov, S., Murikinati, S., Yang, H., Tracey, K.J., Bendszus, M., Rossetti, G., Nawroth, P.P., Bierhaus, A. & Schwaninger, M. (2008) The HMGB1 receptor RAGE mediates ischemic brain damage. *J Neurosci*, 28, 12023-12031.

Muller, S., Ronfani, L. & Bianchi, M.E. (2004) Regulated expression and subcellular localization of HMGB1, a chromatin protein with a cytokine function. *J Intern Med*, 255, 332-343.

Munger, B.L. & Ide, C. (1988) The structure and function of cutaneous sensory receptors. *Archives of histology and cytology*, 51, 1-34.

Muraki, S., Akune, T., Oka, H., Mabuchi, A., En-Yo, Y., Yoshida, M., Saika, A., Nakamura, K., Kawaguchi, H. & Yoshimura, N. (2009) Association of occupational activity with radiographic knee osteoarthritis and lumbar spondylosis in elderly patients of population-based cohorts: a large-scale population-based study. *Arthritis Rheum*, 61, 779-786.

Muzio, M., Ni, J., Feng, P. & Dixit, V.M. (1997) IRAK (Pelle) family member IRAK-2 and MyD88 as proximal mediators of IL-1 signaling. *Science*, 278, 1612-1615.

Nagatani, G., Nomoto, M., Takano, H., Ise, T., Kato, K., Imamura, T., Izumi, H., Makishima, K. & Kohno, K. (2001) Transcriptional activation of the human HMG1 gene in cisplatin-resistant human cancer cells. *Cancer research*, 61, 1592-1597.

Naghavi, M.H., Nowak, P., Andersson, J., Sonnerborg, A., Yang, H., Tracey, K.J. & Vahlne, A. (2003) Intracellular high mobility group B1 protein (HMGB1) represses HIV-1 LTR-directed transcription in a promoter- and cell-specific manner. *Virology*, 314, 179-189.

Nahin, R.L., Ren, K., De Leon, M. & Ruda, M. (1994) Primary sensory neurons exhibit altered gene expression in a rat model of neuropathic pain. *Pain*, 58, 95-108.

Nair, S.M., Zhao, Z., Chou, D.K., Tobet, S.A. & Jungalwala, F.B. (1998) Expression of HNK-1 carbohydrate and its binding protein, SBP-1, in apposing cell surfaces in cerebral cortex and cerebellum. *Neuroscience*, 85, 759-771.

Neugebauer, V., Schaible, H.G. & Schmidt, R.F. (1989) Sensitization of articular afferents to mechanical stimuli by bradykinin. *Pflugers Archiv : European journal of physiology*, 415, 330-335.

Newton, R.A., Bingham, S., Case, P.C., Sanger, G.J. & Lawson, S.N. (2001) Dorsal root ganglion neurons show increased expression of the calcium channel alpha2delta-1 subunit following partial sciatic nerve injury. *Brain research. Molecular brain research*, 95, 1-8.

Nickel, W. & Rabouille, C. (2009) Mechanisms of regulated unconventional protein secretion. *Nature reviews. Molecular cell biology*, 10, 148-155.

Nicol, G.D., Lopshire, J.C. & Pafford, C.M. (1997a) Tumor necrosis factor enhances the capsaicin sensitivity of rat sensory neurons. *J Neurosci*, 17, 975-982.

Nicol, G.D., Vasko, M.R. & Evans, A.R. (1997b) Prostaglandins suppress an outward potassium current in embryonic rat sensory neurons. *J Neurophysiol*, 77, 167-176.

Nissenbaum, J., Devor, M., Seltzer, Z., Gebauer, M., Michaelis, M., Tal, M., Dorfman, R., Abitbul-Yarkoni, M., Lu, Y., Elahipanah, T., delCanho, S., Minert, A., Fried, K., Persson, A.K., Shpigler, H., Shabo, E., Yakir, B., Pisante, A. & Darvasi, A. (2010) Susceptibility to chronic pain following nerve injury is genetically affected by CACNG2. *Genome research*, 20, 1180-1190.

Nitzan-Luques, A., Devor, M. & Tal, M. (2011) Genotype-selective phenotypic switch in primary afferent neurons contributes to neuropathic pain. *Pain*, 152, 2413-2426.

Noguchi, K., Kawai, Y., Fukuoka, T., Senba, E. & Miki, K. (1995) Substance P induced by peripheral nerve injury in primary afferent sensory neurons and its effect on dorsal column nucleus neurons. *J Neurosci*, 15, 7633-7643.

Nowicki, M., Muller, K., Serke, H., Kosacka, J., Vilser, C., Ricken, A. & Spanel-Borowski, K. (2010) Oxidized low-density lipoprotein (oxLDL)-induced cell death in dorsal root ganglion cell cultures depends not on the lectin-like oxLDL receptor-1 but on the toll-like receptor-4. *Journal of neuroscience research*, 88, 403-412.

O'Connor, K.A., Hansen, M.K., Rachal Pugh, C., Deak, M.M., Biedenkapp, J.C., Milligan, E.D., Johnson, J.D., Wang, H., Maier, S.F., Tracey, K.J. & Watkins, L.R. (2003) Further characterization of high mobility group box 1 (HMGB1) as a proinflammatory cytokine: central nervous system effects. *Cytokine*, 24, 254-265.

Obata, K., Katsura, H., Miyoshi, K., Kondo, T., Yamanaka, H., Kobayashi, K., Dai, Y., Fukuoka, T., Akira, S. & Noguchi, K. (2008) Toll-like receptor 3 contributes to spinal glial activation and tactile allodynia after nerve injury. *J Neurochem*, 105, 2249-2259.

Obata, K., Yamanaka, H., Fukuoka, T., Yi, D., Tokunaga, A., Hashimoto, N., Yoshikawa, H. & Noguchi, K. (2003) Contribution of injured and uninjured dorsal root ganglion neurons to pain behavior and the changes in gene expression following chronic constriction injury of the sciatic nerve in rats. *Pain*, 101, 65-77.

Ochoa-Cortes, F., Ramos-Lomas, T., Miranda-Morales, M., Spreadbury, I., Ibeakanma, C., Barajas-Lopez, C. & Vanner, S. (2010) Bacterial cell products signal to mouse colonic nociceptive dorsal root ganglia neurons. *Am J Physiol Gastrointest Liver Physiol*, 299, G723-732.

Ogawa, Y., Aizawa, S., Shirakawa, H. & Yoshida, M. (1995) Stimulation of transcription accompanying relaxation of chromatin structure in cells overexpressing high mobility group 1 protein. *J Biol Chem*, 270, 9272-9280.

Oh, S.B., Tran, P.B., Gillard, S.E., Hurley, R.W., Hammond, D.L. & Miller, R.J. (2001) Chemokines and glycoprotein120 produce pain hypersensitivity by directly exciting primary nociceptive neurons. *J Neurosci*, 21, 5027-5035.

Oh, Y.J., Youn, J.H., Ji, Y., Lee, S.E., Lim, K.J., Choi, J.E. & Shin, J.S. (2009) HMGB1 is phosphorylated by classical protein kinase C and is secreted by a calcium-dependent mechanism. *J Immunol*, 182, 5800-5809.

Ohnishi, M., Katsuki, H., Fukutomi, C., Takahashi, M., Motomura, M., Fukunaga, M., Matsuoka, Y., Isohama, Y., Izumi, Y., Kume, T., Inoue, A. & Akaike, A. (2011) HMGB1 inhibitor glycyrrhizin attenuates intracerebral hemorrhage-induced injury in rats. *Neuropharmacology*, 61, 975-980.

Ohnuma, K., Yamochi, T., Uchiyama, M., Nishibashi, K., Iwata, S., Hosono, O., Kawasaki, H., Tanaka, H., Dang, N.H. & Morimoto, C. (2005) CD26 mediates dissociation of Tollip and IRAK-1 from caveolin-1 and induces upregulation of CD86 on antigen-presenting cells. *Mol Cell Biol*, 25, 7743-7757.

Okamoto, K., Martin, D.P., Schmelzer, J.D., Mitsui, Y. & Low, P.A. (2001a) Pro- and anti-inflammatory cytokine gene expression in rat sciatic nerve chronic constriction injury model of neuropathic pain. *Experimental neurology*, 169, 386-391.

Okamoto, M., Baba, H., Goldstein, P.A., Higashi, H., Shimoji, K. & Yoshimura, M. (2001b) Functional reorganization of sensory pathways in the rat spinal dorsal horn following peripheral nerve injury. *The Journal of physiology*, 532, 241-250.

Okun, E., Griffioen, K.J. & Mattson, M.P. (2011) Toll-like receptor signaling in neural plasticity and disease. *Trends Neurosci*, 34, 269-281.

Olausson, H., Lamarre, Y., Backlund, H., Morin, C., Wallin, B.G., Starck, G., Ekholm, S., Strigo, I., Worsley, K., Vallbo, A.B. & Bushnell, M.C. (2002) Unmyelinated tactile afferents signal touch and project to insular cortex. *Nat Neurosci*, 5, 900-904.

Ono, K., Xu, S., Hitomi, S. & Inenaga, K. (2012) Comparison of the electrophysiological and immunohistochemical properties of acutely dissociated and 1-day cultured rat trigeminal ganglion neurons. *Neurosci Lett*, 523, 162-166.

Oppenheim, J.J. & Yang, D. (2005) Alarmins: chemotactic activators of immune responses. *Curr Opin Immunol*, 17, 359-365.

Ossipov, M.H., Lai, J. & Porreca, F. (2006) Mechanisms of experimental neuropathic pain: integration from animal models *Wall and Melzack's Textbook of Pain*. Elsevier, Churchill Livingstone, pp. 929-946.

Osterloh, J.M., Yang, J., Rooney, T.M., Fox, A.N., Adalbert, R., Powell, E.H., Sheehan, A.E., Avery, M.A., Hackett, R., Logan, M.A., MacDonald, J.M., Ziegenfuss, J.S., Milde, S., Hou, Y.J., Nathan, C., Ding, A., Brown, R.H., Jr., Conforti, L., Coleman, M., Tessier-Lavigne, M., Zuchner, S. & Freeman, M.R. (2012) dSarm/Sarm1 is required for activation of an injury-induced axon death pathway. *Science*, 337, 481-484.

Otoshi, K., Kikuchi, S., Kato, K., Sekiguchi, M. & Konno, S. (2011a) Anti-HMGB1 neutralization antibody improves pain-related behavior induced by application of autologous nucleus pulposus onto nerve roots in rats. *Spine (Phila Pa 1976)*, 36, E692-698.

Otoshi, K., Kikuchi, S., Kato, K., Sekiguchi, M. & Konno, S. (2011b) Anti-HMGB1 neutralization antibody improves pain-related behavior induced by application of autologous nucleus pulposus onto nerve roots in rats. *Spine (Phila Pa 1976)*, 36, E692-698.

Palsson-McDermott, E.M., Doyle, S.L., McGettrick, A.F., Hardy, M., Husebye, H., Banahan, K., Gong, M., Golenbock, D., Espevik, T. & O'Neill, L.A. (2009) TAG, a splice variant of the adaptor TRAM, negatively regulates the adaptor MyD88-independent TLR4 pathway. *Nat Immunol*, 10, 579-586.

Palumbo, R., De Marchis, F., Pusterla, T., Conti, A., Alessio, M. & Bianchi, M.E. (2009) Src family kinases are necessary for cell migration induced by extracellular HMGB1. *J Leukoc Biol*, 86, 617-623.

Palumbo, R., Galvez, B.G., Pusterla, T., De Marchis, F., Cossu, G., Marcu, K.B. & Bianchi, M.E. (2007) Cells migrating to sites of tissue damage in response to the danger signal HMGB1 require NF-kappaB activation. *The Journal of cell biology*, 179, 33-40.

Palumbo, R., Sampaolesi, M., De Marchis, F., Tonlorenzi, R., Colombetti, S., Mondino, A., Cossu, G. & Bianchi, M.E. (2004) Extracellular HMGB1, a signal of tissue damage, induces mesoangioblast migration and proliferation. *The Journal of cell biology*, 164, 441-449.

Park, B.S., Song, D.H., Kim, H.M., Choi, B.S., Lee, H. & Lee, J.O. (2009) The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex. *Nature*, 458, 1191-1195.

Park, J.S., Arcaroli, J., Yum, H.K., Yang, H., Wang, H., Yang, K.Y., Choe, K.H., Strassheim, D., Pitts, T.M., Tracey, K.J. & Abraham, E. (2003) Activation of gene expression in human neutrophils by high mobility group box 1 protein. *Am J Physiol Cell Physiol*, 284, C870-879.

Park, J.S., Svetkauskaite, D., He, Q., Kim, J.Y., Strassheim, D., Ishizaka, A. & Abraham, E. (2004) Involvement of toll-like receptors 2 and 4 in cellular activation by high mobility group box 1 protein. *J Biol Chem*, 279, 7370-7377.

Parkkinen, J. & Rauvala, H. (1991) Interactions of plasminogen and tissue plasminogen activator (t-PA) with amphoterin. Enhancement of t-PA-catalyzed plasminogen activation by amphoterin. *J Biol Chem*, 266, 16730-16735.

Passalacqua, M., Patrone, M., Picotti, G.B., Del Rio, M., Sparatore, B., Melloni, E. & Pontremoli, S. (1998) Stimulated astrocytes release high-mobility group 1 protein, an inducer of LAN-5 neuroblastoma cell differentiation. *Neuroscience*, 82, 1021-1028.

Patel, A.J., Honore, E., Maingret, F., Lesage, F., Fink, M., Duprat, F. & Lazdunski, M. (1998) A mammalian two pore domain mechano-gated S-like K+ channel. *EMBO J*, 17, 4283-4290.

Pedrazzi, M., Averna, M., Sparatore, B., Patrone, M., Salamino, F., Marcoli, M., Maura, G., Cervetto, C., Frattaroli, D., Pontremoli, S. & Melloni, E. (2012) Potentiation of NMDA Receptor-Dependent Cell Responses by Extracellular High Mobility Group Box 1 Protein. *PloS one*, 7, e44518.

Pedrazzi, M., Patrone, M., Passalacqua, M., Ranzato, E., Colamassaro, D., Sparatore, B., Pontremoli, S. & Melloni, E. (2007) Selective proinflammatory activation of astrocytes by high-mobility group box 1 protein signaling. *J Immunol*, 179, 8525-8532.

Peier, A.M., Moqrich, A., Hergarden, A.C., Reeve, A.J., Andersson, D.A., Story, G.M., Earley, T.J., Dragoni, I., McIntyre, P., Bevan, S. & Patapoutian, A. (2002) A TRP channel that senses cold stimuli and menthol. *Cell*, 108, 705-715.

Penzo, M., Molteni, R., Suda, T., Samaniego, S., Raucci, A., Habiel, D.M., Miller, F., Jiang, H.P., Li, J., Pardi, R., Palumbo, R., Olivotto, E., Kew, R.R., Bianchi, M.E. & Marcu, K.B. (2010) Inhibitor of NF-kappa B kinases alpha and beta are both essential for high mobility group box 1-mediated chemotaxis [corrected]. *J Immunol*, 184, 4497-4509.

Perl, E.R. (2007) Ideas about pain, a historical view. *Nature reviews. Neuroscience*, 8, 71-80.

Perrin, F.E., Lacroix, S., Aviles-Trigueros, M. & David, S. (2005) Involvement of monocyte chemoattractant protein-1, macrophage inflammatory protein-1alpha and interleukin-1beta in Wallerian degeneration. *Brain*, 128, 854-866.

Ploeger, B., Mensinga, T., Sips, A., Seinen, W., Meulenbelt, J. & DeJongh, J. (2001) The pharmacokinetics of glycyrrhizic acid evaluated by physiologically based pharmacokinetic modeling. *Drug metabolism reviews*, 33, 125-147.

Pobezinskaya, Y.L., Kim, Y.S., Choksi, S., Morgan, M.J., Li, T., Liu, C. & Liu, Z. (2008) The function of TRADD in signaling through tumor necrosis factor receptor 1 and TRIF-dependent Toll-like receptors. *Nat Immunol*, 9, 1047-1054.

Poltorak, A., He, X., Smirnova, I., Liu, M.Y., Van Huffel, C., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., Freudenberg, M., Ricciardi-Castagnoli, P., Layton, B. & Beutler, B. (1998) Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science*, 282, 2085-2088.

Portenoy, R.K., Payne, D. & Jacobsen, P. (1999) Breakthrough pain: characteristics and impact in patients with cancer pain. *Pain*, 81, 129-134.

Price, D.D. (2000) Psychological and neural mechanisms of the affective dimension of pain. *Science*, 288, 1769-1772.

Pullerits, R., Bokarewa, M., Dahlberg, L. & Tarkowski, A. (2005) Decreased levels of soluble receptor for advanced glycation end products in patients with rheumatoid arthritis indicating deficient inflammatory control. *Arthritis research & therapy*, 7, R817-824.

Qi, J., Buzas, K., Fan, H., Cohen, J.I., Wang, K., Mont, E., Klinman, D., Oppenheim, J.J. & Howard, O.M. (2011) Painful pathways induced by TLR stimulation of dorsal root ganglion neurons. *J Immunol*, 186, 6417-6426.

Qin, J., Qian, Y., Yao, J., Grace, C. & Li, X. (2005a) SIGIRR inhibits interleukin-1 receptor- and toll-like receptor 4-mediated signaling through different mechanisms. *J Biol Chem*, 280, 25233-25241.

Qin, L., Li, G., Qian, X., Liu, Y., Wu, X., Liu, B., Hong, J.S. & Block, M.L. (2005b) Interactive role of the toll-like receptor 4 and reactive oxygen species in LPS-induced microglia activation. *Glia*, 52, 78-84.

Qin, X., Hou, L. & Wang, X. (2004) Lipopolysaccharide evoked peptide release by calcium-induced calcium release. *Neuroreport*, 15, 1003-1006.

Qiu, J., Nishimura, M., Wang, Y., Sims, J.R., Qiu, S., Savitz, S.I., Salomone, S. & Moskowitz, M.A. (2008) Early release of HMGB-1 from neurons after the onset of brain ischemia. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism*, 28, 927-938.

Qiu, J., Xu, J., Zheng, Y., Wei, Y., Zhu, X., Lo, E.H., Moskowitz, M.A. & Sims, J.R. (2010) High-Mobility Group Box 1 Promotes Metalloproteinase-9 Upregulation Through Toll-Like Receptor 4 After Cerebral Ischemia. *Stroke*.

Rajan, B., Polydefkis, M., Hauer, P., Griffin, J.W. & McArthur, J.C. (2003) Epidermal reinnervation after intracutaneous axotomy in man. *The Journal of comparative neurology*, 457, 24-36.

Ramasamy, R., Yan, S.F. & Schmidt, A.M. (2011) Receptor for AGE (RAGE): signaling mechanisms in the pathogenesis of diabetes and its complications. *Ann N Y Acad Sci*, 1243, 88-102.

Raucci, A., Cugusi, S., Antonelli, A., Barabino, S.M., Monti, L., Bierhaus, A., Reiss, K., Saftig, P. & Bianchi, M.E. (2008) A soluble form of the receptor for advanced glycation endproducts (RAGE) is produced by proteolytic cleavage of the membrane-bound form by the sheddase a disintegrin and metalloprotease 10 (ADAM10). *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, 22, 3716-3727.

Rauvala, H. & Pihlaskari, R. (1987) Isolation and some characteristics of an adhesive factor of brain that enhances neurite outgrowth in central neurons. *J Biol Chem*, 262, 16625-16635.

Rauvala, H. & Rouhiainen, A. (2007) RAGE as a receptor of HMGB1 (Amphoterin): roles in health and disease. *Curr Mol Med*, 7, 725-734.

Rauvala, H. & Rouhiainen, A. (2010) Physiological and pathophysiological outcomes of the interactions of HMGB1 with cell surface receptors. *Biochim Biophys Acta*, 1799, 164-170.

Read, C.M., Cary, P.D., Crane-Robinson, C., Driscoll, P.C. & Norman, D.G. (1993) Solution structure of a DNA-binding domain from HMG1. *Nucleic acids research*, 21, 3427-3436.

Rehli, M., Poltorak, A., Schwarzfischer, L., Krause, S.W., Andreesen, R. & Beutler, B. (2000) PU.1 and interferon consensus sequence-binding protein regulate the myeloid expression of the human Toll-like receptor 4 gene. *J Biol Chem*, 275, 9773-9781.

Ren, P.C., Zhang, Y., Zhang, X.D., An, L.J., Lv, H.G., He, J., Gao, C.J. & Sun, X.D. (2012) High-mobility group box 1 contributes to mechanical allodynia and spinal astrocytic activation in a mouse model of type 2 diabetes. *Brain research bulletin*, 88, 332-337.

Rexed, B. (1952) The cytoarchitectonic organization of the spinal cord in the cat. *The Journal of comparative neurology*, 96, 414-495.

Richardson, J.D. & Vasko, M.R. (2002) Cellular mechanisms of neurogenic inflammation. *J Pharmacol Exp Ther*, 302, 839-845.

Rigaud, M., Gemes, G., Weyker, P.D., Cruikshank, J.M., Kawano, T., Wu, H.E. & Hogan, Q.H. (2009) Axotomy depletes intracellular calcium stores in primary sensory neurons. *Anesthesiology*, 111, 381-392.

Ro, L.S., Chen, S.T., Tang, L.M. & Jacobs, J.M. (1999) Effect of NGF and anti-NGF on neuropathic pain in rats following chronic constriction injury of the sciatic nerve. *Pain*, 79, 265-274.

Rodin, B.E. & Kruger, L. (1984) Deafferentation in animals as a model for the study of pain: an alternative hypothesis. *Brain Res*, 319, 213-228.

Rodriguez Parkitna, J., Korostynski, M., Kaminska-Chowaniec, D., Obara, I., Mika, J., Przewlocka, B. & Przewlocki, R. (2006) Comparison of gene expression profiles in neuropathic and inflammatory pain. *Journal of physiology and pharmacology : an official journal of the Polish Physiological Society*, 57, 401-414.

Roger, T., Miconnet, I., Schiesser, A.L., Kai, H., Miyake, K. & Calandra, T. (2005) Critical role for Ets, AP-1 and GATA-like transcription factors in regulating mouse Toll-like receptor 4 (Tlr4) gene expression. *The Biochemical journal*, 387, 355-365.

Rolls, A., Shechter, R., London, A., Ziv, Y., Ronen, A., Levy, R. & Schwartz, M. (2007) Toll-like receptors modulate adult hippocampal neurogenesis. *Nature cell biology*, 9, 1081-1088.

Romero-Calvo, I., Ocon, B., Martinez-Moya, P., Suarez, M.D., Zarzuelo, A., Martinez-Augustin, O. & de Medina, F.S. (2010) Reversible Ponceau staining as a loading control alternative to actin in Western blots. *Analytical biochemistry*, 401, 318-320.

Ruparel, N.B., Patwardhan, A.M., Akopian, A.N. & Hargreaves, K.M. (2008) Homologous and heterologous desensitization of capsaicin and mustard oil responses utilize different cellular pathways in nociceptors. *Pain*, 135, 271-279.

Sahu, D., Debnath, P., Takayama, Y. & Iwahara, J. (2008) Redox properties of the A-domain of the HMGB1 protein. *FEBS letters*, 582, 3973-3978.

Saito, K., Kikuchi, T. & Yoshida, M. (1999) The mechanism of sequence non-specific DNA binding of HMG1/2-box B in HMG1 with DNA. *Protein engineering*, 12, 235-242.

Saito, O., Svensson, C.I., Buczynski, M.W., Wegner, K., Hua, X.Y., Codeluppi, S., Schaloske, R.H., Deems, R.A., Dennis, E.A. & Yaksh, T.L. (2010) Spinal glial TLR4-mediated nociception and production of prostaglandin E(2) and TNF. *British journal of pharmacology*, 160, 1754-1764.

Sakaguchi, M., Murata, H., Yamamoto, K., Ono, T., Sakaguchi, Y., Motoyama, A., Hibino, T., Kataoka, K. & Huh, N.H. (2011) TIRAP, an adaptor protein for TLR2/4, transduces a signal from RAGE phosphorylated upon ligand binding. *PloS one*, 6, e23132.

Sandkuhler, J. (2007) Understanding LTP in pain pathways. *Mol Pain*, 3, 9.

Sandkuhler, J. (2009) Models and mechanisms of hyperalgesia and allodynia. *Physiological reviews*, 89, 707-758.

Sappington, P.L., Yang, R., Yang, H., Tracey, K.J., Delude, R.L. & Fink, M.P. (2002) HMGB1 B box increases the permeability of Caco-2 enterocytic monolayers and impairs intestinal barrier function in mice. *Gastroenterology*, 123, 790-802.

Sato, J. & Perl, E.R. (1991) Adrenergic excitation of cutaneous pain receptors induced by peripheral nerve injury. *Science*, 251, 1608-1610.

Sato, K., Kiyama, H., Park, H.T. & Tohyama, M. (1993) AMPA, KA and NMDA receptors are expressed in the rat DRG neurones. *Neuroreport*, 4, 1263-1265.

Saxena, S. & Caroni, P. (2007) Mechanisms of axon degeneration: from development to disease. *Progress in neurobiology*, 83, 174-191.

Sayyah, M., Javad-Pour, M. & Ghazi-Khansari, M. (2003) The bacterial endotoxin lipopolysaccharide enhances seizure susceptibility in mice: involvement of proinflammatory factors: nitric oxide and prostaglandins. *Neuroscience*, 122, 1073-1080.

Scaffidi, P., Misteli, T. & Bianchi, M.E. (2002) Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. *Nature*, 418, 191-195.

Schafer, M., Fruttiger, M., Montag, D., Schachner, M. & Martini, R. (1996) Disruption of the gene for the myelin-associated glycoprotein improves axonal regrowth along myelin in C57BL/Wlds mice. *Neuron*, 16, 1107-1113.

Schafers, M., Lee, D.H., Brors, D., Yaksh, T.L. & Sorkin, L.S. (2003) Increased sensitivity of injured and adjacent uninjured rat primary sensory neurons to exogenous tumor necrosis factor-alpha after spinal nerve ligation. *J Neurosci*, 23, 3028-3038.

Scheel, O., Papavlassopoulos, M., Blunck, R., Gebert, A., Hartung, T., Zahringer, U., Seydel, U. & Schromm, A.B. (2006) Cell activation by ligands of the toll-like receptor and interleukin-1 receptor family depends on the function of the large-conductance potassium channel MaxiK in human macrophages. *Infection and immunity*, 74, 4354-4356.

Schiraldi, M., Raucci, A., Munoz, L.M., Livoti, E., Celona, B., Venereau, E., Apuzzo, T., De Marchis, F., Pedotti, M., Bachi, A., Thelen, M., Varani, L., Mellado, M., Proudfoot, A., Bianchi, M.E. & Uguccioni, M. (2012) HMGB1 promotes recruitment of inflammatory cells to damaged tissues by forming a complex with CXCL12 and signaling via CXCR4. *The Journal of experimental medicine*, 209, 551-563.

Schmidt, R., Schmelz, M., Forster, C., Ringkamp, M., Torebjork, E. & Handwerker, H. (1995) Novel classes of responsive and unresponsive C nociceptors in human skin. *J Neurosci*, 15, 333-341.

Schmued, L.C. & Hopkins, K.J. (2000) Fluoro-Jade B: a high affinity fluorescent marker for the localization of neuronal degeneration. *Brain Res*, 874, 123-130.

Scholz, J. & Woolf, C.J. (2007) The neuropathic pain triad: neurons, immune cells and glia. *Nat Neurosci*, 10, 1361-1368.

Schweizer, A., Feige, U., Fontana, A., Muller, K. & Dinarello, C.A. (1988) Interleukin-1 enhances pain reflexes. Mediation through increased prostaglandin E2 levels. *Agents and actions*, 25, 246-251.

Seijffers, R., Allchorne, A.J. & Woolf, C.J. (2006) The transcription factor ATF-3 promotes neurite outgrowth. *Mol Cell Neurosci*, 32, 143-154.

Seijffers, R., Mills, C.D. & Woolf, C.J. (2007) ATF3 increases the intrinsic growth state of DRG neurons to enhance peripheral nerve regeneration. *J Neurosci*, 27, 7911-7920.

Seltzer, Z., Dubner, R. & Shir, Y. (1990) A novel behavioral model of neuropathic pain disorders produced in rats by partial sciatic nerve injury. *Pain*, 43, 205-218.

Seydel, U., Scheel, O., Muller, M., Brandenburg, K. & Blunck, R. (2001) A K+ channel is involved in LPS signaling. *J Endotoxin Res*, 7, 243-247.

Sha, Y., Zmijewski, J., Xu, Z. & Abraham, E. (2008) HMGB1 develops enhanced proinflammatory activity by binding to cytokines. *J Immunol*, 180, 2531-2537.

Shamash, S., Reichert, F. & Rotshenker, S. (2002) The cytokine network of Wallerian degeneration: tumor necrosis factor-alpha, interleukin-1alpha, and interleukin-1beta. *J Neurosci*, 22, 3052-3060.

Shechter, R., Ronen, A., Rolls, A., London, A., Bakalash, S., Young, M.J. & Schwartz, M. (2008) Toll-like receptor 4 restricts retinal progenitor cell proliferation. *The Journal of cell biology*, 183, 393-400.

Shen, Y.J., DeBellard, M.E., Salzer, J.L., Roder, J. & Filbin, M.T. (1998) Myelin-associated glycoprotein in myelin and expressed by Schwann cells inhibits axonal regeneration and branching. *Mol Cell Neurosci*, 12, 79-91.

Shi, T.J., Tandrup, T., Bergman, E., Xu, Z.Q., Ulfhake, B. & Hokfelt, T. (2001) Effect of peripheral nerve injury on dorsal root ganglion neurons in the C57 BL/6J mouse: marked changes both in cell numbers and neuropeptide expression. *Neuroscience*, 105, 249-263.

Shibasaki, M., Sasaki, M., Miura, M., Mizukoshi, K., Ueno, H., Hashimoto, S., Tanaka, Y. & Amaya, F. (2010) Induction of high mobility group box-1 in dorsal root ganglion contributes to pain hypersensitivity after peripheral nerve injury. *Pain*, 149, 514-521.

Shields, S.D., Eckert, W.A., 3rd & Basbaum, A.I. (2003) Spared nerve injury model of neuropathic pain in the mouse: a behavioral and anatomic analysis. *J Pain*, 4, 465-470.

Shimazu, R., Akashi, S., Ogata, H., Nagai, Y., Fukudome, K., Miyake, K. & Kimoto, M. (1999) MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. *J Exp Med*, 189, 1777-1782.

Shimura, H., Nitahara, A., Ito, A., Tomiyama, K., Ito, M. & Kawai, K. (2005) Up-regulation of cell surface Toll-like receptor 4-MD2 expression on dendritic epidermal T cells after the emigration from epidermis during cutaneous inflammation. *Journal of dermatological science*, 37, 101-110.

Shinder, V. & Devor, M. (1994) Structural basis of neuron-to-neuron cross-excitation in dorsal root ganglia. *Journal of neurocytology*, 23, 515-531.

Shortland, P., Woolf, C.J. & Fitzgerald, M. (1989) Morphology and somatotopic organization of the central terminals of hindlimb hair follicle afferents in the rat lumbar spinal cord. *The Journal of comparative neurology*, 289, 416-433.

Shortland, P.J., Baytug, B., Krzyzanowska, A., McMahon, S.B., Priestley, J.V. & Averill, S. (2006) ATF3 expression in L4 dorsal root ganglion neurons after L5 spinal nerve transection. *Eur J Neurosci*, 23, 365-373.

Shu, X. & Mendell, L.M. (1999) Nerve growth factor acutely sensitizes the response of adult rat sensory neurons to capsaicin. *Neurosci Lett*, 274, 159-162.

Shu, X. & Mendell, L.M. (2001) Acute sensitization by NGF of the response of small-diameter sensory neurons to capsaicin. *J Neurophysiol*, 86, 2931-2938.

Shubayev, V.I., Angert, M., Dolkas, J., Campana, W.M., Palenscar, K. & Myers, R.R. (2006) TNFalpha-induced MMP-9 promotes macrophage recruitment into injured peripheral nerve. *Mol Cell Neurosci*, 31, 407-415.

Sikandar, S. & Dickenson, A.H. (2012) Visceral pain: the ins and outs, the ups and downs. *Current opinion in supportive and palliative care*, 6, 17-26.

Silverman, J.D. & Kruger, L. (1990) Selective neuronal glycoconjugate expression in sensory and autonomic ganglia: relation of lectin reactivity to peptide and enzyme markers. *Journal of neurocytology*, 19, 789-801.

Sims, G.P., Rowe, D.C., Rietdijk, S.T., Herbst, R. & Coyle, A.J. (2010) HMGB1 and RAGE in inflammation and cancer. *Annu Rev Immunol*, 28, 367-388.

Sitia, G., Iannacone, M., Muller, S., Bianchi, M.E. & Guidotti, L.G. (2007) Treatment with HMGB1 inhibitors diminishes CTL-induced liver disease in HBV transgenic mice. *J Leukoc Biol*, 81, 100-107.

Sivilotti, L. & Woolf, C.J. (1994) The contribution of GABAA and glycine receptors to central sensitization: disinhibition and touch-evoked allodynia in the spinal cord. *J Neurophysiol*, 72, 169-179.

Slack, J.L., Schooley, K., Bonnert, T.P., Mitcham, J.L., Qwarnstrom, E.E., Sims, J.E. & Dower, S.K. (2000) Identification of two major sites in the type I interleukin-1 receptor cytoplasmic region responsible for coupling to pro-inflammatory signaling pathways. *J Biol Chem*, 275, 4670-4678.

Snider, W.D. & McMahon, S.B. (1998) Tackling pain at the source: new ideas about nociceptors. *Neuron*, 20, 629-632.

Sommer, C. & Kress, M. (2004) Recent findings on how proinflammatory cytokines cause pain: peripheral mechanisms in inflammatory and neuropathic hyperalgesia. *Neurosci Lett*, 361, 184-187.

Sommer, C. & Myers, R.R. (1995) Neurotransmitters in the spinal cord dorsal horn in a model of painful neuropathy and in nerve crush. *Acta neuropathologica*, 90, 478-485.

Sommer, C., Petrausch, S., Lindenlaub, T. & Toyka, K.V. (1999) Neutralizing antibodies to interleukin 1-receptor reduce pain associated behavior in mice with experimental neuropathy. *Neurosci Lett*, 270, 25-28.

Sommer, E.W., Kazimierczak, J. & Droz, B. (1985) Neuronal subpopulations in the dorsal root ganglion of the mouse as characterized by combination of ultrastructural and cytochemical features. *Brain Res*, 346, 310-326.

Song, M.J., Hwang, S., Wong, W., Round, J., Martinez-Guzman, D., Turpaz, Y., Liang, J., Wong, B., Johnson, R.C., Carey, M. & Sun, R. (2004) The DNA architectural protein HMGB1 facilitates RTA-mediated viral gene expression in gamma-2 herpesviruses. *Journal of virology*, 78, 12940-12950.

Song, P.I., Park, Y.M., Abraham, T., Harten, B., Zivony, A., Neparidze, N., Armstrong, C.A. & Ansel, J.C. (2002) Human keratinocytes express functional CD14 and toll-like receptor 4. *The Journal of investigative dermatology*, 119, 424-432.

Song, X.J., Hu, S.J., Greenquist, K.W., Zhang, J.M. & LaMotte, R.H. (1999) Mechanical and thermal hyperalgesia and ectopic neuronal discharge after chronic compression of dorsal root ganglia. *J Neurophysiol*, 82, 3347-3358.

Song, Y., Li, H.M., Xie, R.G., Yue, Z.F., Song, X.J., Hu, S.J. & Xing, J.L. (2012) Evoked bursting in injured Abeta dorsal root ganglion neurons: a mechanism underlying tactile allodynia. *Pain*, 153, 657-665.

Sorge, R.E., LaCroix-Fralish, M.L., Tuttle, A.H., Sotocinal, S.G., Austin, J.S., Ritchie, J., Chanda, M.L., Graham, A.C., Topham, L., Beggs, S., Salter, M.W. & Mogil, J.S. (2011) Spinal cord Toll-like receptor 4 mediates inflammatory and neuropathic hypersensitivity in male but not female mice. *J Neurosci*, 31, 15450-15454.

Sorkin, L.S., Xiao, W.H., Wagner, R. & Myers, R.R. (1997) Tumour necrosis factor-alpha induces ectopic activity in nociceptive primary afferent fibres. *Neuroscience*, 81, 255-262.

Sparatore, B., Patrone, M., Passalacqua, M., Pedrazzi, M., Gaggero, D., Pontremoli, S. & Melloni, E. (2001) Extracellular processing of amphoterin generates a peptide active on erythroleukaemia cell differentiation. *The Biochemical journal*, 357, 569-574.

Stemkowski, P.L. & Smith, P.A. (2012) Long-term IL-1beta exposure causes subpopulation-dependent alterations in rat dorsal root ganglion neuron excitability. *J Neurophysiol*, 107, 1586-1597.

Straino, S., Di Carlo, A., Mangoni, A., De Mori, R., Guerra, L., Maurelli, R., Panacchia, L., Di Giacomo, F., Palumbo, R., Di Campli, C., Uccioli, L., Biglioli, P., Bianchi, M.E., Capogrossi, M.C. & Germani, A. (2008) High-mobility group box 1 protein in human and murine skin: involvement in wound healing. *The Journal of investigative dermatology*, 128, 1545-1553.

Stros, M. (2010) HMGB proteins: interactions with DNA and chromatin. *Biochim Biophys Acta*, 1799, 101-113.

Stros, M., Launholt, D. & Grasser, K.D. (2007) The HMG-box: a versatile protein domain occurring in a wide variety of DNA-binding proteins. *Cellular and molecular life sciences : CMLS*, 64, 2590-2606.

Study, R.E. & Kral, M.G. (1996) Spontaneous action potential activity in isolated dorsal root ganglion neurons from rats with a painful neuropathy. *Pain*, 65, 235-242.

Sufka, K.J. (1994) Conditioned place preference paradigm: a novel approach for analgesic drug assessment against chronic pain. *Pain*, 58, 355-366.

Suzuki, N., Suzuki, S., Duncan, G.S., Millar, D.G., Wada, T., Mirtsos, C., Takada, H., Wakeham, A., Itie, A., Li, S., Penninger, J.M., Wesche, H., Ohashi, P.S., Mak, T.W. & Yeh, W.C. (2002) Severe impairment of interleukin-1 and Toll-like receptor signalling in mice lacking IRAK-4. *Nature*, 416, 750-756.

Swett, J.E. & Woolf, C.J. (1985) The somatotopic organization of primary afferent terminals in the superficial laminae of the dorsal horn of the rat spinal cord. *The Journal of comparative neurology*, 231, 66-77.

Tabeta, K., Yamazaki, K., Akashi, S., Miyake, K., Kumada, H., Umemoto, T. & Yoshie, H. (2000) Toll-like receptors confer responsiveness to lipopolysaccharide from Porphyromonas gingivalis in human gingival fibroblasts. *Infection and immunity*, 68, 3731-3735.

Tabuchi, M., Imamura, S., Kawakami, Z., Ikarashi, Y. & Kase, Y. (2012) The Blood-Brain Barrier Permeability of 18beta-Glycyrrhetinic Acid, a Major Metabolite of Glycyrrhizin in Glycyrrhiza Root, a Constituent of the Traditional Japanese Medicine Yokukansan. *Cellular and molecular neurobiology*.

Takata, K., Kitamura, Y., Kakimura, J., Shibagaki, K., Tsuchiya, D., Taniguchi, T., Smith, M.A., Perry, G. & Shimohama, S. (2003) Role of high mobility group protein-1 (HMG1) in amyloid-beta homeostasis. *Biochem Biophys Res Commun*, 301, 699-703.

Tal, M., Wall, P.D. & Devor, M. (1999) Myelinated afferent fiber types that become spontaneously active and mechanosensitive following nerve transection in the rat. *Brain Res*, 824, 218-223.

Tandrup, T., Woolf, C.J. & Coggeshall, R.E. (2000) Delayed loss of small dorsal root ganglion cells after transection of the rat sciatic nerve. *The Journal of comparative neurology*, 422, 172-180.

Tang, D., Kang, R., Cheh, C.W., Livesey, K.M., Liang, X., Schapiro, N.E., Benschop, R., Sparvero, L.J., Amoscato, A.A., Tracey, K.J., Zeh, H.J. & Lotze, M.T. (2010a) HMGB1 release and redox regulates autophagy and apoptosis in cancer cells. *Oncogene*, 29, 5299-5310.

Tang, D., Loze, M.T., Zeh, H.J. & Kang, R. (2010b) The redox protein HMGB1 regulates cell death and survival in cancer treatment. *Autophagy*, 6, 1181-1183.

Tang, S.C., Arumugam, T.V., Xu, X., Cheng, A., Mughal, M.R., Jo, D.G., Lathia, J.D., Siler, D.A., Chigurupati, S., Ouyang, X., Magnus, T., Camandola, S. & Mattson, M.P. (2007) Pivotal role for neuronal Toll-like receptors in ischemic brain injury and functional deficits. *Proc Natl Acad Sci U S A*, 104, 13798-13803.

Tanga, F.Y., Nutile-McMenemy, N. & DeLeo, J.A. (2005) The CNS role of Toll-like receptor 4 in innate neuroimmunity and painful neuropathy. *Proc Natl Acad Sci U S A*, 102, 5856-5861.

Tanga, F.Y., Raghavendra, V. & DeLeo, J.A. (2004) Quantitative real-time RT-PCR assessment of spinal microglial and astrocytic activation markers in a rat model of neuropathic pain. *Neurochemistry international*, 45, 397-407.

Tanimura, N., Saitoh, S., Matsumoto, F., Akashi-Takamura, S. & Miyake, K. (2008) Roles for LPS-dependent interaction and relocation of TLR4 and TRAM in TRIF-signaling. *Biochem Biophys Res Commun*, 368, 94-99.

Taskinen, H.S. & Roytta, M. (2000) Increased expression of chemokines (MCP-1, MIP-1alpha, RANTES) after peripheral nerve transection. *Journal of the peripheral nervous system : JPNS*, 5, 75-81.

Thieblemont, N. & Wright, S.D. (1999) Transport of bacterial lipopolysaccharide to the golgi apparatus. *J Exp Med*, 190, 523-534.

Todd, A.J. (2010) Neuronal circuitry for pain processing in the dorsal horn. *Nature reviews. Neuroscience*, 11, 823-836.

Tong, W., Wang, W., Huang, J., Ren, N., Wu, S.X. & Li, Y.Q. (2010) Spinal high-mobility group box 1 contributes to mechanical allodynia in a rat model of bone cancer pain. *Biochemical and biophysical research communications*, 395, 572-576.

Toshchakov, V., Jones, B.W., Perera, P.Y., Thomas, K., Cody, M.J., Zhang, S., Williams, B.R., Major, J., Hamilton, T.A., Fenton, M.J. & Vogel, S.N. (2002) TLR4, but not TLR2, mediates IFN-beta-induced STAT1alpha/beta-dependent gene expression in macrophages. *Nat Immunol*, 3, 392-398.

Toth, C., Lander, J. & Wiebe, S. (2009) The prevalence and impact of chronic pain with neuropathic pain symptoms in the general population. *Pain Med*, 10, 918-929.

Treede, R.D., Jensen, T.S., Campbell, J.N., Cruccu, G., Dostrovsky, J.O., Griffin, J.W., Hansson, P., Hughes, R., Nurmikko, T. & Serra, J. (2008) Neuropathic pain: redefinition and a grading system for clinical and research purposes. *Neurology*, 70, 1630-1635.

Treede, R.D., Meyer, R.A., Raja, S.N. & Campbell, J.N. (1995) Evidence for two different heat transduction mechanisms in nociceptive primary afferents innervating monkey skin. *The Journal of physiology*, 483 (Pt 3), 747-758.

Triantafilou, M., Lepper, P.M., Briault, C.D., Ahmed, M.A., Dmochowski, J.M., Schumann, C. & Triantafilou, K. (2008) Chemokine receptor 4 (CXCR4) is part of the lipopolysaccharide "sensing apparatus". *European journal of immunology*, 38, 192-203.

Truini, A., Padua, L., Biasiotta, A., Caliandro, P., Pazzaglia, C., Galeotti, F., Inghilleri, M. & Cruccu, G. (2009) Differential involvement of A-delta and A-beta fibres in neuropathic pain related to carpal tunnel syndrome. *Pain*, 145, 105-109.

Tsan, M.F. (2011) Heat shock proteins and high mobility group box 1 protein lack cytokine function. *J Leukoc Biol*.

Tsuda, M., Inoue, K. & Salter, M.W. (2005) Neuropathic pain and spinal microglia: a big problem from molecules in "small" glia. *Trends Neurosci*, 28, 101-107.

Tsujino, H., Kondo, E., Fukuoka, T., Dai, Y., Tokunaga, A., Miki, K., Yonenobu, K., Ochi, T. & Noguchi, K. (2000) Activating transcription factor 3 (ATF3) induction by axotomy in sensory and motoneurons: A novel neuronal marker of nerve injury. *Mol Cell Neurosci*, 15, 170-182.

Tsung, A., Klune, J.R., Zhang, X., Jeyabalan, G., Cao, Z., Peng, X., Stolz, D.B., Geller, D.A., Rosengart, M.R. & Billiar, T.R. (2007) HMGB1 release induced by liver ischemia involves Toll-like receptor 4 dependent reactive oxygen species production and calcium-mediated signaling. *J Exp Med*, 204, 2913-2923.

Tulleuda, A., Cokic, B., Callejo, G., Saiani, B., Serra, J. & Gasull, X. (2011) TRESK channel contribution to nociceptive sensory neurons excitability: modulation by nerve injury. *Mol Pain*, 7, 30.

Turk, D. & Okifuji, A. (2009) Pain terms and taxonomies of pain. In SM, F., JC, B., JP, R. (eds) *Bonica's Management of Pain*. Lippincott Williams & Wilkins, New York, NY, USA, pp. 13-23.

Turk, D.C., Wilson, H.D. & Cahana, A. (2011) Treatment of chronic non-cancer pain. *Lancet*, 377, 2226-2235.

Ueda, T. & Yoshida, M. (2010) HMGB proteins and transcriptional regulation. *Biochim Biophys Acta*, 1799, 114-118.

Uramoto, H., Izumi, H., Nagatani, G., Ohmori, H., Nagasue, N., Ise, T., Yoshida, T., Yasumoto, K. & Kohno, K. (2003) Physical interaction of tumour suppressor p53/p73 with CCAAT-binding transcription factor 2 (CTF2) and differential regulation of human high-mobility group 1 (HMG1) gene expression. *The Biochemical journal*, 371, 301-310.

Vallieres, N., Berard, J.L., David, S. & Lacroix, S. (2006) Systemic injections of lipopolysaccharide accelerates myelin phagocytosis during Wallerian degeneration in the injured mouse spinal cord. *Glia*, 53, 103-113.

Vasko, M.R., Guo, C. & Kelley, M.R. (2005) The multifunctional DNA repair/redox enzyme Ape1/Ref-1 promotes survival of neurons after oxidative stress. *DNA repair*, 4, 367-379.

Venereau, E., Casalgrandi, M., Schiraldi, M., Antoine, D.J., Cattaneo, A., De Marchis, F., Liu, J., Antonelli, A., Preti, A., Raeli, L., Shams, S.S., Yang, H., Varani, L., Andersson, U., Tracey, K.J., Bachi, A., Uguccioni, M. & Bianchi, M.E. (2012) Mutually exclusive redox forms of HMGB1 promote cell recruitment or proinflammatory cytokine release. *J Exp Med*.

Verdu, B., Decosterd, I., Buclin, T., Stiefel, F. & Berney, A. (2008) Antidepressants for the treatment of chronic pain. *Drugs*, 68, 2611-2632.

Verge, V.M., Richardson, P.M., Benoit, R. & Riopelle, R.J. (1989) Histochemical characterization of sensory neurons with high-affinity receptors for nerve growth factor. *Journal of neurocytology*, 18, 583-591.

Vetter, I. & Lewis, R.J. (2010) Characterization of endogenous calcium responses in neuronal cell lines. *Biochemical pharmacology*, 79, 908-920.

Vezzani, A., Maroso, M., Balosso, S., Sanchez, M.A. & Bartfai, T. (2011) IL-1 receptor/Toll-like receptor signaling in infection, inflammation, stress and neurodegeneration couples hyperexcitability and seizures. *Brain Behav Immun*, 25, 1281-1289.

Vezzoli, M., Castellani, P., Campana, L., Corna, G., Bosurgi, L., Manfredi, A.A., Bianchi, M.E., Rubartelli, A. & Rovere-Querini, P. (2010) Redox remodeling: a candidate regulator of HMGB1 function in injured skeletal muscle. *Ann N Y Acad Sci*, 1209, 83-90.

Vezzoli, M., Castellani, P., Corna, G., Castiglioni, A., Bosurgi, L., Monno, A., Brunelli, S., Manfredi, A.A., Rubartelli, A. & Rovere-Querini, P. (2011) High-mobility group box 1 release and redox regulation accompany regeneration and remodeling of skeletal muscle. *Antioxidants & redox signaling*, 15, 2161-2174.

Vincent, A.M., Perrone, L., Sullivan, K.A., Backus, C., Sastry, A.M., Lastoskie, C. & Feldman, E.L. (2007) Receptor for advanced glycation end products activation injures primary sensory neurons via oxidative stress. *Endocrinology*, 148, 548-558.

Vo, T., Rice, A.S. & Dworkin, R.H. (2009) Non-steroidal anti-inflammatory drugs for neuropathic pain: how do we explain continued widespread use? *Pain*, 143, 169-171.

von Hehn, C.A., Baron, R. & Woolf, C.J. (2012) Deconstructing the neuropathic pain phenotype to reveal neural mechanisms. *Neuron*, 73, 638-652.

Wadachi, R. & Hargreaves, K.M. (2006) Trigeminal Nociceptors Express TLR-4 and CD14: a Mechanism for Pain due to Infection. *J Dent Res*, 85, 49-53.

Wald, D., Qin, J., Zhao, Z., Qian, Y., Naramura, M., Tian, L., Towne, J., Sims, J.E., Stark, G.R. & Li, X. (2003) SIGIRR, a negative regulator of Toll-like receptor-interleukin 1 receptor signaling. *Nat Immunol*, 4, 920-927.

Wall, P.D. & Devor, M. (1983) Sensory afferent impulses originate from dorsal root ganglia as well as from the periphery in normal and nerve injured rats. *Pain*, 17, 321-339.

Wall, P.D., Devor, M., Inbal, R., Scadding, J.W., Schonfeld, D., Seltzer, Z. & Tomkiewicz, M.M. (1979a) Autotomy following peripheral nerve lesions: experimental anaesthesia dolorosa. *Pain*, 7, 103-111.

Wall, P.D. & Gutnick, M. (1974) Properties of afferent nerve impulses originating from a neuroma. *Nature*, 248, 740-743.

Wall, P.D., Scadding, J.W. & Tomkiewicz, M.M. (1979b) The production and prevention of experimental anesthesia dolorosa. *Pain*, 6, 175-182.

Wang, D., Lou, J., Ouyang, C., Chen, W., Liu, Y., Liu, X., Cao, X., Wang, J. & Lu, L. (2010) Ras-related protein Rab10 facilitates TLR4 signaling by promoting replenishment of TLR4 onto the plasma membrane. *Proc Natl Acad Sci U S A*, 107, 13806-13811.

Wang, H., Bloom, O., Zhang, M., Vishnubhakat, J.M., Ombrellino, M., Che, J., Frazier, A., Yang, H., Ivanova, S., Borovikova, L., Manogue, K.R., Faist, E., Abraham, E., Andersson, J., Andersson, U., Molina, P.E., Abumrad, N.N., Sama, A. & Tracey, K.J. (1999) HMG-1 as a late mediator of endotoxin lethality in mice. *Science*, 285, 248-251.

Wang, Q., Ding, Q., Zhou, Y., Gou, X., Hou, L., Chen, S., Zhu, Z. & Xiong, L. (2009) Ethyl pyruvate attenuates spinal cord ischemic injury with a wide therapeutic window through inhibiting high-mobility group box 1 release in rabbits. *Anesthesiology*, 110, 1279-1286.

Wang, T., Molliver, D.C., Jing, X., Schwartz, E.S., Yang, F.C., Samad, O.A., Ma, Q. & Davis, B.M. (2011a) Phenotypic switching of nonpeptidergic cutaneous sensory neurons following peripheral nerve injury. *PloS one*, 6, e28908.

Wang, X., Loram, L.C., Ramos, K., de Jesus, A.J., Thomas, J., Cheng, K., Reddy, A., Somogyi, A.A., Hutchinson, M.R., Watkins, L.R. & Yin, H. (2012a) Morphine activates neuroinflammation in a manner parallel to endotoxin. *Proc Natl Acad Sci U S A*.

Wang, Y., Chen, T., Han, C., He, D., Liu, H., An, H., Cai, Z. & Cao, X. (2007) Lysosome-associated small Rab GTPase Rab7b negatively regulates TLR4 signaling in macrophages by promoting lysosomal degradation of TLR4. *Blood*, 110, 962-971.

Wang, Y., Wilson, S.M., Brittain, J.M., Ripsch, M.S., Salome, C., Park, K.D., White, F.A., Khanna, R. & Kohn, H. (2011b) Merging Structural Motifs of Functionalized Amino Acids and alpha-Aminoamides Results in Novel Anticonvulsant Compounds with Significant Effects on Slow and Fast Inactivation of Voltage-gated Sodium Channels and in the Treatment of Neuropathic Pain. *ACS Chem Neurosci*, 2, 317-322.

Wang, Y., Yang, Y., Liu, X., Wang, N., Cao, H., Lu, Y., Zhou, H. & Zheng, J. (2012b) Inhibition of clathrin/dynamin-dependent internalization interferes with LPS-mediated TRAM-TRIF-dependent signaling pathway. *Cellular immunology*, 274, 121-129.

Watkins, L.R. & Maier, S.F. (2002) Beyond neurons: evidence that immune and glial cells contribute to pathological pain states. *Physiological reviews*, 82, 981-1011.

Watson, M.B., Costello, D.A., Carney, D.G., McQuillan, K. & Lynch, M.A. (2010) SIGIRR modulates the inflammatory response in the brain. *Brain Behav Immun*, 24, 985-995.

Weir, H.M., Kraulis, P.J., Hill, C.S., Raine, A.R., Laue, E.D. & Thomas, J.O. (1993) Structure of the HMG box motif in the B-domain of HMG1. *EMBO J*, 12, 1311-1319.

Wells, M.R. & Vaidya, U. (1989) Morphological alterations in dorsal root ganglion neurons after peripheral axon injury: association with changes in metabolism. *Experimental neurology*, 104, 32-38.

White, F.A., Bhangoo, S.K. & Miller, R.J. (2005a) Chemokines: integrators of pain and inflammation. *Nature reviews. Drug discovery*, 4, 834-844.

White, F.A., Sun, J., Waters, S.M., Ma, C., Ren, D., Ripsch, M., Steflik, J., Cortright, D.N., Lamotte, R.H. & Miller, R.J. (2005b) Excitatory monocyte chemoattractant protein-1 signaling is up-regulated in sensory neurons after chronic compression of the dorsal root ganglion. *Proc Natl Acad Sci U S A*, 102, 14092-14097.

Whitmore, M.M., Iparraguirre, A., Kubelka, L., Weninger, W., Hai, T. & Williams, B.R. (2007) Negative regulation of TLR-signaling pathways by activating transcription factor-3. *J Immunol*, 179, 3622-3630.

Willis, W.D. (2002) Long-term potentiation in spinothalamic neurons. *Brain research. Brain research reviews*, 40, 202-214.

Wong-Riley, M.T. & Kageyama, G.H. (1986) Localization of cytochrome oxidase in the mammalian spinal cord and dorsal root ganglia, with quantitative analysis of ventral horn cells in monkeys. *The Journal of comparative neurology*, 245, 41-61.

Woolf, C.J. (1983) Evidence for a central component of post-injury pain hypersensitivity. *Nature*, 306, 686-688.

Woolf, C.J. (2010) What is this thing called pain? *J Clin Invest*, 120, 3742-3744.

Woolf, C.J. & King, A.E. (1989) Subthreshold components of the cutaneous mechanoreceptive fields of dorsal horn neurons in the rat lumbar spinal cord. *J Neurophysiol*, 62, 907-916.

Woolf, C.J. & Salter, M.W. (2000) Neuronal plasticity: increasing the gain in pain. *Science*, 288, 1765-1769.

Wright, S.D., Ramos, R.A., Tobias, P.S., Ulevitch, R.J. & Mathison, J.C. (1990) CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science*, 249, 1431-1433.

Wu, F.X., Bian, J.J., Miao, X.R., Huang, S.D., Xu, X.W., Gong, D.J., Sun, Y.M., Lu, Z.J. & Yu, W.F. (2010a) Intrathecal siRNA against Toll-like receptor 4 reduces nociception in a rat model of neuropathic pain. *Int J Med Sci*, 7, 251-259.

Wu, G., Ringkamp, M., Hartke, T.V., Murinson, B.B., Campbell, J.N., Griffin, J.W. & Meyer, R.A. (2001) Early onset of spontaneous activity in uninjured C-fiber nociceptors after injury to neighboring nerve fibers. *J Neurosci*, 21, RC140.

Wu, G., Ringkamp, M., Murinson, B.B., Pogatzki, E.M., Hartke, T.V., Weerahandi, H.M., Campbell, J.N., Griffin, J.W. & Meyer, R.A. (2002) Degeneration of myelinated efferent fibers induces spontaneous activity in uninjured C-fiber afferents. *J Neurosci*, 22, 7746-7753.

Wu, H., Ma, J., Wang, P., Corpuz, T.M., Panchapakesan, U., Wyburn, K.R. & Chadban, S.J. (2010b) HMGB1 contributes to kidney ischemia reperfusion injury. *Journal of the American Society of Nephrology : JASN*, 21, 1878-1890.

Xiao, H.S., Huang, Q.H., Zhang, F.X., Bao, L., Lu, Y.J., Guo, C., Yang, L., Huang, W.J., Fu, G., Xu, S.H., Cheng, X.P., Yan, Q., Zhu, Z.D., Zhang, X., Chen, Z., Han, Z.G. & Zhang, X. (2002) Identification of gene expression profile of dorsal root ganglion in the rat peripheral axotomy model of neuropathic pain. *Proc Natl Acad Sci U S A*, 99, 8360-8365.

Xie, Y., Zhang, J., Petersen, M. & LaMotte, R.H. (1995) Functional changes in dorsal root ganglion cells after chronic nerve constriction in the rat. *J Neurophysiol*, 73, 1811-1820.

Yaksh, T.L. (1989) Behavioral and autonomic correlates of the tactile evoked allodynia produced by spinal glycine inhibition: effects of modulatory receptor systems and excitatory amino acid antagonists. *Pain*, 37, 111-123.

Yamakami, M., Yoshimori, T. & Yokosawa, H. (2003) Tom1, a VHS domain-containing protein, interacts with tollip, ubiquitin, and clathrin. *J Biol Chem*, 278, 52865-52872.

Yamamoto, M., Sato, S., Hemmi, H., Hoshino, K., Kaisho, T., Sanjo, H., Takeuchi, O., Sugiyama, M., Okabe, M., Takeda, K. & Akira, S. (2003) Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. *Science*, 301, 640-643.

Yamamoto, M., Sato, S., Hemmi, H., Sanjo, H., Uematsu, S., Kaisho, T., Hoshino, K., Takeuchi, O., Kobayashi, M., Fujita, T., Takeda, K. & Akira, S. (2002) Essential role for TIRAP in activation of the signalling cascade shared by TLR2 and TLR4. *Nature*, 420, 324-329.

Yamamoto, W., Sugiura, A., Nakazato-Imasato, E. & Kita, Y. (2008) Characterization of primary sensory neurons mediating static and dynamic allodynia in rat chronic constriction injury model. *The Journal of pharmacy and pharmacology*, 60, 717-722.

Yamamura, Y., Santa, T., Kotaki, H., Uchino, K., Sawada, Y. & Iga, T. (1995) Administration-route dependency of absorption of glycyrrhizin in rats: intraperitoneal administration dramatically enhanced bioavailability. *Biological & pharmaceutical bulletin*, 18, 337-341.

Yammani, R.R. (2012) S100 proteins in cartilage: role in arthritis. *Biochim Biophys Acta*, 1822, 600-606.

Yang, D., Chen, Q., Yang, H., Tracey, K.J., Bustin, M. & Oppenheim, J.J. (2007) High mobility group box-1 protein induces the migration and activation of human dendritic cells and acts as an alarmin. *Journal of leukocyte biology*, 81, 59-66.

Yang, D. & Oppenheim, J.J. (2004) Antimicrobial proteins act as "alarmins" in joint immune defense. *Arthritis and rheumatism*, 50, 3401-3403.

Yang, H., Hreggvidsdottir, H.S., Palmblad, K., Wang, H., Ochani, M., Li, J., Lu, B., Chavan, S., Rosas-Ballina, M., Al-Abed, Y., Akira, S., Bierhaus, A., Erlandsson-Harris, H., Andersson, U. & Tracey, K.J. (2010) A critical cysteine is required for HMGB1 binding to Toll-like receptor 4 and activation of macrophage cytokine release. *Proc Natl Acad Sci U S A*, 107, 11942-11947.

Yang, H., Lundback, P., Ottosson, L., Erlandsson-Harris, H., Venereau, E., Bianchi, M.E., Al-Abed, Y., Andersson, U., Tracey, K.J. & Antoine, D.J. (2011) Redox modification of cysteine residues regulates the cytokine activity of HMGB1. *Molecular medicine*.

Yang, H., Ochani, M., Li, J., Qiang, X., Tanovic, M., Harris, H.E., Susarla, S.M., Ulloa, L., Wang, H., DiRaimo, R., Czura, C.J., Roth, J., Warren, H.S., Fink, M.P., Fenton, M.J., Andersson, U. & Tracey, K.J. (2004) Reversing established sepsis with antagonists of endogenous high-mobility group box 1. *Proc Natl Acad Sci U S A*, 101, 296-301.

Yonekura, H., Yamamoto, Y., Sakurai, S., Petrova, R.G., Abedin, M.J., Li, H., Yasui, K., Takeuchi, M., Makita, Z., Takasawa, S., Okamoto, H., Watanabe, T. & Yamamoto, H. (2003) Novel splice variants of the receptor for advanced glycation end-products expressed in human vascular endothelial cells and pericytes, and their putative roles in diabetes-induced vascular injury. *The Biochemical journal*, 370, 1097-1109.

Youn, J.H., Oh, Y.J., Kim, E.S., Choi, J.E. & Shin, J.S. (2008) High mobility group box 1 protein binding to lipopolysaccharide facilitates transfer of lipopolysaccharide to CD14 and enhances lipopolysaccharide-mediated TNF-alpha production in human monocytes. *J Immunol*, 180, 5067-5074.

Youn, J.H. & Shin, J.S. (2006) Nucleocytoplasmic shuttling of HMGB1 is regulated by phosphorylation that redirects it toward secretion. *J Immunol*, 177, 7889-7897.

Yu, L., Wang, L. & Chen, S. (2010) Endogenous toll-like receptor ligands and their biological significance. *J Cell Mol Med*, 14, 2592-2603.

Yuk, J.M., Yang, C.S., Shin, D.M., Kim, K.K., Lee, S.K., Song, Y.J., Lee, H.M., Cho, C.H., Jeon, B.H. & Jo, E.K. (2009) A dual regulatory role of apurinic/apyrimidinic endonuclease 1/redox factor-1 in HMGB1-induced inflammatory responses. *Antioxidants & redox signaling*, 11, 575-588.

Zappavigna, V., Falciola, L., Helmer-Citterich, M., Mavilio, F. & Bianchi, M.E. (1996) HMG1 interacts with HOX proteins and enhances their DNA binding and transcriptional activation. *EMBO J*, 15, 4981-4991.

Zarember, K.A. & Godowski, P.J. (2002) Tissue expression of human Toll-like receptors and differential regulation of Toll-like receptor mRNAs in leukocytes in response to microbes, their products, and cytokines. *J Immunol*, 168, 554-561.

Zelenka, M., Schafers, M. & Sommer, C. (2005) Intraneural injection of interleukin-1beta and tumor necrosis factor-alpha into rat sciatic nerve at physiological doses induces signs of neuropathic pain. *Pain*, 116, 257-263.

Zhang, G. & Ghosh, S. (2002) Negative regulation of toll-like receptor-mediated signaling by Tollip. *J Biol Chem*, 277, 7059-7065.

Zhang, J.M., Donnelly, D.F., Song, X.J. & Lamotte, R.H. (1997) Axotomy increases the excitability of dorsal root ganglion cells with unmyelinated axons. *J Neurophysiol*, 78, 2790-2794.

Zhang, J.M., Song, X.J. & LaMotte, R.H. (1999) Enhanced excitability of sensory neurons in rats with cutaneous hyperalgesia produced by chronic compression of the dorsal root ganglion. *J Neurophysiol*, 82, 3359-3366.

Zhang, N., Inan, S., Cowan, A., Sun, R., Wang, J.M., Rogers, T.J., Caterina, M. & Oppenheim, J.J. (2005) A proinflammatory chemokine, CCL3, sensitizes the heat- and capsaicin-gated ion channel TRPV1. *Proc Natl Acad Sci U S A*, 102, 4536-4541.

Zhang, Q., O'Hearn, S., Kavalukas, S.L. & Barbul, A. (2012) Role of high mobility group box 1 (HMGB1) in wound healing. *The Journal of surgical research*, 176, 343-347.

Zhang, S.J., Buchthal, B., Lau, D., Hayer, S., Dick, O., Schwaninger, M., Veltkamp, R., Zou, M., Weiss, U. & Bading, H. (2011) A signaling cascade of nuclear calcium-CREB-ATF3 activated by synaptic NMDA receptors defines a gene repression module that protects against extrasynaptic NMDA receptor-induced neuronal cell death and ischemic brain damage. *J Neurosci*, 31, 4978-4990.

Zhang, X., Wheeler, D., Tang, Y., Guo, L., Shapiro, R.A., Ribar, T.J., Means, A.R., Billiar, T.R., Angus, D.C. & Rosengart, M.R. (2008) Calcium/calmodulin-dependent protein kinase (CaMK) IV mediates nucleocytoplasmic shuttling and release of HMGB1 during lipopolysaccharide stimulation of macrophages. *J Immunol*, 181, 5015-5023.

Zhao, X., Kuja-Panula, J., Rouhiainen, A., Chen, Y.C., Panula, P. & Rauvala, H. (2011) High mobility group box-1 (HMGB1; amphoterin) is required for zebrafish brain development. *J Biol Chem*, 286, 23200-23213.

Zhao, Z., Chou, D.K., Nair, S.M., Tobet, S. & Jungalwala, F.B. (2000) Expression of sulfoglucuronyl (HNK-1) carbohydrate and its binding protein (SBP-1) in developing rat cerebellum. *Brain research. Developmental brain research*, 120, 165-180.

Zhou, Z., Yamamoto, Y., Sugai, F., Yoshida, K., Kishima, Y., Sumi, H., Nakamura, H. & Sakoda, S. (2004) Hepatoma-derived growth factor is a neurotrophic factor harbored in the nucleus. *J Biol Chem*, 279, 27320-27326.

Zhu, Y.F. & Henry, J.L. (2012) Excitability of Abeta sensory neurons is altered in an animal model of peripheral neuropathy. *BMC neuroscience*, 13, 15.

Zochodne, D.W., Allison, J.A., Ho, W., Ho, L.T., Hargreaves, K. & Sharkey, K.A. (1995) Evidence for CGRP accumulation and activity in experimental neuromas. *The American journal of physiology*, 268, H584-590.

Zochodne, D.W. & Ho, L.T. (1992) Hyperemia of injured peripheral nerve: sensitivity to CGRP antagonism. *Brain Res*, 598, 59-66.

Zochodne, D.W., Levy, D., Zwiers, H., Sun, H., Rubin, I., Cheng, C. & Lauritzen, M. (1999) Evidence for nitric oxide and nitric oxide synthase activity in proximal stumps of transected peripheral nerves. *Neuroscience*, 91, 1515-1527.

Zylka, M.J., Rice, F.L. & Anderson, D.J. (2005) Topographically distinct epidermal nociceptive circuits revealed by axonal tracers targeted to Mrgprd. *Neuron*, 45, 17-25.

CURRICULUM VITAE

Polina Feldman

EDUCATION

- August 2008- February 2013
  Indiana University, Indianapolis, IN
  Medical Neuroscience Graduate Program
  Doctor of Philosophy

- August 2003- May 2007
  University of Wisconsin, Madison, WI
  Neurobiology, Psychology
  Bachelor of Science

PUBLICATIONS

1. **Feldman P**, Due MR, Ripsch MS, Khanna R and White FA (2012). The persistent release of HMGB1 contributes to tactile hyperalgesia in a rodent model of neuropathic pain. *Journal of NeuroInflammation*. 9(1):180.

2. Due MR*, Piekarz AD*, Wilson N, **Feldman P**, Ripsch MS, Chavez S, Yin H, Khanna R, and White FA (2012). Neuroexcitatory effects of morphine-3-glucuronide are dependent on Toll-like receptor 4 signaling. *Journal of NeuroInflammation*. 9(1):200.

3. Toyoda R, Assimacopoulos S, Wilcoxon J, Taylor A, **Feldman P**, Suzuki-Hirano A, Shimogori T, and Grove EA (2010). FGF8 acts as a classic diffusible morphogen to pattern the neocortex. *Development*. 137: 3439-3448.

4. Caronia G, Wilcoxon J, **Feldman P**, and Grove EA (2010). BMP signaling in the telencephalon controls development of the hippocampal dentate gyrus and regulates fear-related behavior. *Journal of Neuroscience*. 30(18):6291-6301.

5. White FA, **Feldman P**, and Miller RJ (2009). Chemokine signaling and the management of neuropathic pain. *Molecular Interventions*. 9(4): 188-195.

ABSTRACTS AND PRESENTATIONS

1. **Feldman P**, Due MR, Cheon BM, Ripsch MS, and White FA (2011). Peripheral nerve injury-induced tactile hyperalgesia is dependent on SDF1a/CXCR4 interactions in the rat. 41st Annual Meeting of the Society for Neuroscience. Washington, DC. *(presenter)*

2. Due MR, Wilson N, **Feldman P**, Yin H, and White FA (2011). Increased functional expression of TLR4 in sensory neurons following repeated morphine treatment contributes to opioid-induced hyperalgesia. 41st Annual Meeting of the Society for Neuroscience. Washington, DC.

3. **Feldman P**, Due MR, Ripsch MS, and White FA (2011). Regulation of Toll-like receptor 4 signaling in neuropathic pain. 9th Cytokine and Inflammation Meeting. San Diego, CA. *(presenter)*

4. **Feldman P**, Jung H, Ripsch MS, Miller RJ, and White FA (2009). Expression of the chemokine receptor, CCR2, in primary sensory neurons of the adult mouse following peripheral nerve transection and crush injury. 39th Annual Meeting of the Society for Neuroscience, 561.12. Chicago, IL. *(presenter)*

5. Foster RM, McClung C, **Feldman P**, Ripsch MS, Fitzgerald MP, and White FA (2009). Pelvic floor muscle injury-induced tactile hypernociceptive behavior is reversed by the administration of CXCR4 antagonist, AMD3100. 39th Annual Meeting of the Society for Neuroscience, 655.6. Chicago, IL.

6. Caronia G, Wilcoxon JS, **Feldman P**, and Grove EA (2008). BMP signaling is required for dentate gyrus development and adult neurogenesis. 38th Annual Meeting of the Society for Neuroscience, 122.3 San Diego, CA.

7. Wilcoxon JS, **Feldman P**, and Grove EA (2008). Deletion of fibroblast growth factor receptors disrupts the cortical area map. 38th Annual Meeting of the Society for Neuroscience, 816.1. San Diego, CA.

8. Wilcoxon JS, **Feldman P**, and Grove EA (2007). Cortical area boundaries shift following genetic manipulations of FGF8/17 signaling. 37th Annual Meeting of the Society for Neuroscience, 561.15. San Diego, CA.

SCHOLARSHIPS AND AWARDS

- Kepner Award for Research Excellence, 2012
- TEDMED Frontline Scholarship, 2012
- GSO Education Enhancement Grant for Travel, 2010

RESEARCH EXPERIENCE

- Indiana University School of Medicine, Indianapolis, IN (2009-2013)
  Medical Neuroscience Graduate Program- *Thesis Project*
  Mentor- Fletcher A. White, Ph.D
  The role of high mobility group box 1(HMGB1) and toll-like receptor 4 (TLR4) in a rodent model of neuropathic pain

- Loyola University-Chicago, Maywood, IL (2008- 2009)
  Neuroscience Graduate Program- *Rotation*
  Advisor- Fletcher A. White, Ph.D
  Expression of the chemokine receptor, CCR2, in primary sensory neurons following peripheral nerve transection and crush injury

- University of Chicago, Chicago, IL (2007-2008)
  Department of Neurobiology- *Research Technician*
  Principal Investigator- Elizabeth A. Grove, Ph.D
  FGF8 acts as a classic diffusible morphogen to pattern the neocortex

- University of Wisconsin, Madison, WI (2006-2007)
  Department of Cellular & Molecular Neuroscience- *Laboratory Technician*
  Principal Investigator- Albee Messing, V.M.D, Ph.D
  Assessed rosenthal fiber protein inclusions in Alexander disease-associated glial fibrillary acidic protein transgenic mice

- University of Wisconsin, Madison, WI (2005-2007)
  Department of Affective Neuroscience- *Research Assistant*
  Principal Investigator- Richard Davidson, Ph.D
  Evaluation of Mindfulness Based Stress Reduction (MBSR) and Health Enhancement Program in normal controls

PROFESSIONAL AFFILIATIONS

- International Association for the Study of Pain (IASP) student member, 2011-2013
- Society for Neuroscience (SFN), student member, 2009-2013