THE ROLE OF DORSAL ROOT GANGLION GLUTAMINASE IN ACUTE AND CHRONIC INFLAMMATORY PAIN

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THE ROLE OF DORSAL ROOT GANGLION GLUTAMINASE IN ACUTE AND CHRONIC INFLAMMATORY PAIN

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Abstract:

Scope and methods of the study.

The purpose of this study was to provide further evidence to support the concept that dorsal root ganglion (DRG) glutaminase, the synthetic enzyme for the neurotransmitter glutamate, is a potential target for analgesic therapeutics. Behavioral study, fluorescent microscopy and quantitative image analysis were used to investigate the temporal expression of DRG glutaminase after peripheral inflammation. The role of tonic glutaminase and glutamate in initiating acute and chronic inflammatory pain was also explored.

Findings and Conclusions.

Significant elevation of glutaminase was observed in subpopulations of DRG neurons that were labeled with nociception-related neuropeptide markers in small- to medium-sized neuronal cell bodies. Elevated glutaminase was also found in peripheral nerve during acute phase of inflammation. These results indicated that after inflammation, elevated glutaminase was synthesized in the neuronal cell bodies and then transported to the peripheral nerve terminals around the inflamed site, where glutamate was produced and released contributing to peripheral sensitization. Inhibition of glutaminase at the peripheral nerve terminal prior to inflammation 1) achieved robust long-term anti-edema and anti-nociceptive effects; 2) inhibited the inflammation-induced elevation of glutaminase in the peptidergic DRG neuronal cell bodies. These results supported the notion that after the onset of inflammation, tonic or early glutamate production at peripheral nerve terminal had a “feed-forward” mechanism by up-regulating glutaminase synthesis in DRG neurons. It suggested that early treatment that targeted the acute glutamate production at the peripheral terminal might prevent the development of chronic pain and/or provide more effective alleviation if the pain continues into the chronic phase with the pathology. With further understanding of the role of dorsal root ganglion glutaminase in acute and chronic inflammatory pain, it was rational to propose that glutaminase and glutamate metabolism can be novel potential targets for analgesic therapeutics.

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ABBREVIATIONS

AIA	adjuvant-induced arthritis
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
CFA	complete Freund's adjuvant
CGRP	calcitonin gene-related peptide
DON	6-diazo-5-oxo-L-norleucine
EAAT	excitatory amino acid transporter
EAAC1 (EAAT3)	excitatory amino acid carrier 1
GLAST (EAAT1)	glutamate aspartate transporter
GLN	glutamine
GLS	glutaminase
GLT-1 (EAAT2)	glutamate type 1 transporter
GLU	glutamate
GluR	glutamate receptor
KA	kainic acid
NGF	nerve growth factor
NMDA	N-methyl-D-aspartic acid
TrkA	neurotrophic tyrosine kinase, receptor, type 1
TRPV 1	transient receptor potential cation channel subfamily v member 1
SNAT	Na+-coupled neutral amino acid transporter
SP	substance P
VGLUT	vesicular glutamate transporter

CHAPTER I

INTRODUCTION

I. Cytology of primary sensory neurons

Size, myelination, and conduction velocity

The sensation of pain is detected and mediated by a specialized neural pathway. Primary afferent neurons are the first order neurons in transducing thermal, mechanical and chemical stimuli and relaying the somatosensory information to the thalamus and primary somatosensory cortex in the brain via the dorsal horn of the spinal cord or brainstem. Primary afferents that innervate the face and body arise from neuronal cell bodies in trigeminal and dorsal root ganglion (DRG), respectively. Primary afferent neurons are pseudounipolar neurons, and the axon of each neuron splits into two branches. One branch projects peripherally to innervate the target tissues, such as skin, joint and muscle; the other branch projects centrally to the spinal cord or brainstem. Studies have demonstrated there is a general correlation between DRG neuronal cell body size, axon size and conduction velocity. This means that large sized neurons have large diameter axons and fast conduction velocity, and small neurons have small axons and slow conduction velocity (Yoshida and Matsuda, 1979, Lee et al., 1986). The cell bodies of the rat primary sensory neurons are broadly classified by size (cross-sectional area or diameter): large (>800 μm^2, 32 μm), medium (400-800 μm^2, 23-32 μm) and small (<400 μm^2, 23 μm) (Fang et al., 2005, Hoffman et al., 2010). These neurons can be further classified

as Aα/β-, Aδ-, or C- fibers on the basis of conduction velocity and myelination. Large DRG neurons have heavily myelinated Aα/β-fibers with fast conduction velocity (Aα: 80-120 m/s; Aβ: 35-75 m/s) and carry sensory information such as vibration, fine touch and proprioception to the central nervous system. Medium DRG neurons have lightly myelinated Aδ-fibers and slow conduction velocity at 5-35 m/s. Small DRG neurons have unmyelinated C- fibers and conduction velocity at 0.5-2.0 m/s. Medium- and small-sized neurons carry the sensations of temperature and pain. The primary sensory neurons that are capable of transducing and encoding noxious stimuli are termed nociceptors (IASP, 1986, Vervest and Schimmel, 1988, Loeser and Treede, 2008). In normal physiological conditions, C- and Aδ- nociceptors have a high threshold for activation and respond only to stimuli that have sufficient energy to potentially or actually cause tissue damage. With the presence of ongoing tissue damage or nerve injury, nociceptive neurons alter their response patterns and become sensitized. Section II will focus on the two major types of sensitization: peripheral and central sensitization.

Neurochemical classification of DRG neurons

Dorsal root ganglion neurons develop from neural crest cells that migrate from the dorsal area of the neural tube, but not all neurons are born at the same time. Several studies show that large neurons are born early and mostly become proprioceptors or low-threshold mechanoreceptors, while small neurons are generated later than the large neurons, and become mainly nociceptors (Snider and McMahon, 1998). The heterogeneity of DRG neurons, i.e., their ability to detect and transduce different sensory stimuli, may be related to differential patterns in their neurochemical coding, e.g., the combination of transducers, neurotransmitters, neuromodulators, receptors, and ion channels that are expressed in the neurons. Efforts have been made to identify cytochemical and electrophysiological markers that characterize the phenotype and function of nociceptive neurons in their physiological and pathological stages (Lawson, 2002). Like other neurons in the nervous system, primary sensory neurons require signaling neurotrophins released by their target tissue for survival during development and for the maintenance of neuronal subpopulations. Using trophic factor dependence and neurochemical expression, nociceptors in adult rat DRGs can be put into two

populations: peptidergic vs. non-peptidergic neurons. The peptidergic neurons express tyrosine kinase receptor A (TrkA), a high-affinity receptor for nerve growth factor (NGF), and these neurons are dependent on NGF from embryonic stage (Otten et al., 1980) to adulthood to maintain their nociceptive phenotype (Priestley et al., 2002). Calcitonin gene-related peptide (CGRP) and substance P (SP) are two neuropeptides found in the peptidergic DRG neurons that have been extensively studied. The non-peptidergic neurons can be labeled with isolectin B4, a lectin that binds to the α-D-galactose carbohydrate residues on cell membranes (Fullmer et al., 2004). The adult non-peptidergic neurons are low in TrkA expression, but express high levels of Ret, the corresponding receptor for glial cell line-derived neurotrophic factor (GDNF) from early postnatal development (Molliver et al., 1997). The peptidergic and non-peptidergic nociceptors also express distinct sets of transducers and ion channels and have distinct peripheral and central targets (Snider and McMahon, 1998, Woolf and Ma, 2007). Various neurochemical markers, therefore, can be identified with immunohistochemisty/immunocytochemical and fluorescent microscopy techniques.

Section summary: 1) DRG neurons are heterogeneous and can be divided into different subpopulations by size, conduction velocity of the axon, sensory modality, and neurochemical characteristics; 2) Most nociceptive neurons have small to medium size cell bodies, while large DRG neurons carry mainly vibration, fine touch and proprioception; 3) Though neuronal cell body size does not strictly reflect the absolute function of each neuron, dividing DRG neurons into different populations by sizes allows us evaluate the alteration in general functional groups rather than evaluating the total alteration of a heterogeneous population; 4) Studies correlating neurochemical characteristics with their sensory properties provide valuable information for identifying different subpopulations of DRG neurons.

II. Nociception and sensitization: plasticity of nociceptors

Pain can be simultaneously protective and debilitating (Woolf, 2010). Patients with analgesia who are not able to respond to painful stimuli often incur severe physical damage by unperceived noxious

stimuli. On the other side, acute and chronic pain can be the result from many sources and causes may beyond tissue damage or greater than the observed tissue damage, leading to decreased the patient's socioeconomic status and the quality of life.

Pain and nociception

Detecting painful stimuli is one of the most important functions carried out by the nervous system. The nervous system processes the warning signs detected by the defense systems when physical integrity is threatened by potential or actual damage. Researchers studying pain often use the word "pain" interchangeably with another word "nociception". The definition and scope of the two words, however, are not identical. According to the International Association for the Study of Pain (IASP) taxonomy, pain is defined as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (IASP, 1986). This definition emphasizes the psychological aspect of pain, which is an individual, subjective experience rather than proportional to severity of actual tissue damage or injury. The word "nociception" was introduced to the field to provide a neutral description that directly reflects the neural activity related to a damaging stimulus (IASP, 1986, Vervest and Schimmel, 1988, Loeser and Treede, 2008). When studies involve higher cortical functions or a psychological issue is emphasized, it is recommended to keep these two concepts separate. "Pain" and "nociception" are used interchangeably by most researchers on a more general level and in the current dissertation unless emphasized.

Transducers and the initiation of nociception (brief review of (Woolf and Ma, 2007))

Thermal, mechanical and chemical stimuli are three general categories of somatosensation conveyed by the primary afferents and these stimuli are painful when "sufficiently" intense. How are these stimuli converted to signals that are understandable to the brain? In 1999, David Julius and colleague identified the transient receptor potential vanilloid 1 (TRPV1) protein as the transducer for noxious heat and chemical stimuli and, to date, TRPV1 has been the most thoroughly studied transducer protein expressed by nociceptors. Transducer proteins are high-threshold ion channels by nature, which means that under physiological condition they are activated only when the stimulus is intense

enough. The activation of transducers allows a transient cation influx (sodium and calcium ions), termed as the "transducer potential" by Woolf *et al*., and causes local depolarization of the nociceptive nerve terminal. The transducer potential leads to activation of voltage-gated sodium channels (Nav1.7, 1.8 and 1.9) and produces the "generator potential", which sums as an action potential propagating along the nociceptive axon via Nav1.6, 1.7 and 1.8 sodium channels. At the central terminal of primary sensory neuron axon, depolarization of the presynaptic membrane by the action potential causes calcium influx and the release of glutamate into the synaptic cleft. By binding to the postsynaptic receptors on spinal dorsal horn neurons, glutamate activates the ligand-gated ion channels, resulting in cation (sodium and calcium ions) influx and depolarization of the postsynaptic membrane. Nociceptive information is converted to action potentials and transmitted to the higher order neurons in the nociceptive pathways located in the thalamus and ultimately to somatosensory cortex (Martin, 2012).

The transducer protein for mechanosensation is less clear. Several molecules have been proposed as potential mechanotransduction ion channels, including acid-sensing ion channel (ASIC) 1-3, transient receptor potential ankyrin 1 (TRPA1), and P2X receptors (Dai et al., 2004, Lumpkin et al., 2010). Again, the transducer potential produced by transducers is essential for activation of nociceptors. In summary, under normal condition, these transducer proteins, localized to the nociceptive nerve terminal, are activated during the presence of high-intensity thermal, mechanical and/or chemical stimuli. This type of pain is termed as "nociceptive pain" and serves as a crucial protective mechanism (Woolf, 2010).

Sensitization: not all pains are the same

Pain has three different manifestations: nociceptive pain, inflammatory pain, and pathological pain (Woolf, 2010). In normal conditions, only an intense noxious stimulus is detected and conducted by nociceptors, a specialized subpopulation of primary afferent terminals. This neural response is transient and occurs only with the presence of the actual stimulus. More importantly, there is no alteration in the threshold of nociceptors. This type of pain is termed as "nociceptive pain" (Woolf,

2010). Nociceptive pain serves as an alarm system and is important in maintaining the physical integrity of tissue by preventing inducing tissue damage.

Inflammatory pain and pathological pain occur when there is actual tissue damage or nerve injury. In these conditions, nociceptive neurons decrease their threshold and increase their responsiveness to suprathreshold (painful) stimuli, clinically termed as "hyperalgesia". Sometimes spontaneous discharge occurs and the recruitment of non-nociceptive (or silent nociceptive) neurons responding to subthreshold (non-painful) stimuli, which is referred as allodynia. Hyperalgesia and allodynia are usually observed at the same time. These increased responses are called "sensitization" and they are the result of the plasticity in response properties of the nociceptors or CNS sensory neurons along the pain transmission pathway. Sensitization can be further classified into peripheral sensitization and central sensitization, based on their different locations and mechanisms.

Peripheral sensitization

Other than detecting and conveying sensory information to the central nervous system, primary afferents also have an efferent function to regulate homeostasis at the peripheral target. For example, tissue damage and inflammation cause immediate local depolarization of the nociceptive nerve terminals. Activated peptide-releasing nerve terminals release vasoactive substances such as CGRP and SP and lead to increased arteriole dilation and venular permeability (Holzer, 1998). The increased blood flow and vascular permeability at the site of inflammation activate the innate immune system and recruit innate immune cells, such as neutrophils, mast cells, and macrophages, as well as T lymphocytes from the adaptive immune system (Chiu et al., 2012). These immune cells secrete pro-inflammatory molecules, including serotonin, bradykinin, prostaglandin, histamine, and NGF (Julius and Basbaum, 2001). These pro-inflammatory mediators interact with their cogent receptors on nociceptive peripheral terminals and increase the transducing and/or firing properties of the nociceptors, which is a phenomenon known as "neurogenic inflammation" (Richardson and Vasko, 2002).

Neurogenic inflammation is a double-edged sword that on one side causes sensitization to trigger the guarding behaviors that will facilitate wound healing and immune defense and, on the other side, it is one of the initial mechanisms that lead to chronic peripheral sensitization. The total effects of peripheral sensitization include increased responsiveness and reduced threshold of nociceptive neurons to the stimulation of their receptive field. Some inflammatory mediators, such as protons, ATP and glutamate, can directly depolarize the peripheral nerve terminal. Other inflammatory mediators, such as NGF (Lewin et al., 1993), bind to their tyrosine kinase receptors (Trks) and are retrogradely transported to the neuronal cell body to influence the transcription, translation and trafficking of key proteins, such as CGRP and SP (Lindsay and Harmar, 1989, Woolf et al., 1994).

Central sensitization

The synapses between primary afferents and second order neurons in the spinal cord dorsal horn also are modified in response to sustained noxious signals from the periphery. Central sensitization is defined as "an increase in the excitability of the neurons within the central nervous system," which is characterized by noxious responses to non-noxious stimuli, allodynia (IASP, 1986). Central sensitization can be divided into two phases: early and late. Repeated stimuli within a shorter period of time (early phase) will produce activity-related central sensitization, which is mainly characterized by spontaneous firing of spinal cord neurons. It also can induce the insertion of new AMPA receptors at the postsynaptic membranes of spinal cord neurons, sharing a similar mechanism as long-term potentiation (LTP) (Ji et al., 2003, Latremoliere and Woolf, 2009). If noxious stimuli occur in a long period of time (late phase), transcription and translation signals in spinal cord neurons will be activated by the prolonged stimuli (Ji et al., 2003, Woolf and Ma, 2007, Latremoliere and Woolf, 2009). Both phases of central sensitization are activated by sustained noxious stimuli from primary afferents. Central sensitization, however, can continue in the absence of a peripheral stimulus or after recovery from actual tissue damage, resulting in spontaneous central pain. Central sensitization, therefore, is considered as a molecular basis for chronic pain (Ji et al., 2003, Latremoliere and Woolf, 2009).

Driving forces of phenotypic switch in nociceptors: neuronal activity and neurotrophic factors

The neurochemical phenotype of primary afferents is malleable and can be modified by their own neuronal activity and/or retrograde transport of signaling molecules. The molecular alteration often involves functional neuropeptides, receptors, or ion channels at the levels of transcription, translation, posttranslational modification, and trafficking. These alterations in neurochemical characteristics will lead to the modification of responsiveness of primary afferent neurons to sensory stimuli. Similar phenomena also can occur in "non-nociceptive" neurons and contribute to inflammatory hypersensitivity by altering their phenotype to one resembling pain fibers via modified neurochemical expression. For example, after inflammation some of the low-threshold mechanoreceptors or some silent nociceptors start to express SP and gain the capacity to activate the spinal cord neurons in the nociceptive pathway. This means that the dorsal horn neurons now can be activated by these innocuous stimuli, which will be interpreted as pain by the brain (Woolf and Doubell, 1994, Neumann et al., 1996, Xu et al., 2000, Djouhri and Lawson, 2004). This phenomenon found in the primary sensory neurons has been termed the "phenotypic switch" of nociceptors (Woolf and Ma, 2007).

The peripheral terminals of primary afferents keep constant communication with their neuronal cell bodies in the DRG via electrical signals in the form of action potentials or via retrograde transport of specific signaling molecules, such as the NGF-TrkA complex (Harrington and Ginty, 2013). Altered electrical activity and retrograde transport signaling molecules are also two major activities that "notify" the neuronal cell bodies about inflammatory/pathological conditions to initiate and maintain the phenotypic switch. For example, increased electrical activity within 15 min of complete Freund's adjuvant (CFA) injection causes a rapid transcription of SP and CGRP mRNA in DRG neuronal cell bodies innervating the target tissue. Blockade of this electrical activity with local anesthetic in the sciatic nerve prevents the rapid induction of neuropeptide mRNA expression in DRG neurons (Donaldson et al., 1994, Bulling et al., 2001). These studies show that neuronal discharge may be the early signal to cause the up-regulated transcription of neuropeptide mRNA. The rapid up-regulation

of mRNAs in the neuronal cell body is unlikely due to the retrograde transport of trophic factors because of the relative slow rate of transport. Inflammatory mediators released at the nerve terminal cause peripheral sensitization that includes increased neuronal discharge in nociceptors. Increase in neuronal depolarization in turn may trigger a serial downstream signaling cascade in the neuronal cell bodies and lead to the rapid transcription of mRNA (Donaldson et al., 1994, Bulling et al., 2001, Puehler et al., 2004).

In another study, nerve blockage with local anesthetic diminishes the upregulation of μ-opioid receptor (MOR) mRNA within 8 h, but does not block a delayed elevation present at 96 h after CFA-injection (Puehler et al., 2004). Studies using electrophysiology also show that some of the electrophysiological alterations are completed 2 – 4 d after CFA (Djouhri and Lawson, 1999, Djouhri et al., 2001). These studies indicate that there is a delayed signal(s) that modifies the expression of neuropeptides. This signal travels slower than the neuronal discharge, and arrives later in the neuronal cell bodies. Moreover, studies show decreased neuropeptide expression in the DRG neuronal cell bodies two weeks after peripheral nerve transection (Zhang et al., 1993, Verge et al., 1995), which indicates that this signal is dependent on the integrity of the peripheral axon. This supports the assumption that the delayed elevation of neuropeptides and other neurochemical components is driven by the retrograde transport of a neurotrophic factor. NGF is one of the neurotrophic factors essential for maintaining the physiological functions of primary sensory neurons in development, survival, and normal neuronal function (Snider and McMahon, 1998, Woolf and Ma, 2007). NGF is expressed by target tissues and binds to the TrkA receptors located at the peripheral terminals of primary afferents. At early stages of development, NGF is required for the survival and differentiation of all nociceptive neurons. Rats that are exposed *in utero* to maternal antibodies to nerve growth factor (NGF) show a 70% decrease in the number of DRG neurons (McDougal et al., 1981). Later in the postnatal stage, some nociceptors decrease their expression of TrkA receptors and start to express Ret, the corresponding receptor for glial cell line-derived neurotrophic factor (GDNF) and differentiated into the so-called “non-peptidergic” nociceptors (Molliver et al., 1997, Snider and

McMahon, 1998). NGF remains to be the key neurotrophic factor for peptide-expressing nociceptors. Application of anti-NGF antibody causes a decrease in the expression level of neuropeptides in adult rat DRG neurons, as well as in the number of intradermal fibers at the peripheral target (Lindsay and Harmar, 1989, Hoffman et al., 2011). During inflammation, additional NGF is released from keratinocytes, neutrophils, mast cells and macrophages (Donnerer et al., 1992, Bennett et al., 1998, Albers and Davis, 2007). NGF binds to the high-affinity TrkA receptor, is internalized by endocytosis, and transported back to the neuronal cell body (Stoeckel et al., 1975, Ehlers et al., 1995, Campenot and MacInnis, 2004), where it is considered as the key contributor to the phenotypic switch in peptide-containing nociceptors.

Section summary: 1) The perception of nociception is not static. Neurons that function in the nociceptive pathway undergo plastic alteration during inflammation or nerve injury; 2) Peripheral and central sensitization are two forms of neuronal plasticity in primary afferents and spinal cord dorsal horn; 3) Neuronal activity and neurotrophic factors are two important modulatory signals from the peripheral terminals that keep the cell bodies "notified" of peripheral events. They also direct primary sensory neurons to undergo long-term plastic adaptations when peripheral terminals are challenged by tissue damage or nerve injury.

III. Two neuropeptides as immunohistochemical markers of nociceptive neurons

CGRP and SP are expressed in subpopulations of DRG neurons

The neuropeptides CGRP and SP are considered as major initiators of neurogenic inflammation and as neurochemical markers for nociceptive neurons (Holzer, 1998). They are expressed in the subpopulations of DRG neurons that are highly dependent on NGF during development and adulthood to maintain their phenotype as nociceptive neurons (Otten et al., 1980, Amann et al., 1996). In rodent DRG, CGRP is found in 22% to 45% (Hanesch et al., 1993a, Price and Flores, 2007) and SP is expressed in 15% to 25% of the neuronal cell bodies (Lawson et al., 1997, Price and Flores, 2007). The unequal percentages of the two neuropeptides indicate that CGRP has a larger proportion of total

DRG neurons and suggests a wider peripheral projection compared to SP neurons (Lawson, 1995). Electrophysiological studies in guinea pig DRG neurons show that SP immunoreactivity is limited to nociceptive neurons with cross sectional area < 400 μm^2, while CGRP is mainly expressed small- (< 400 μm^2) and medium- (400-800 μm^2) sized neurons, including a few Aβ-fiber nociceptors (Lawson et al., 1997, Carlton and Hargett, 2002, Lawson, 2002). Both CGRP and SP are extensively co-localized in the rat lumbar DRG and dorsal horn of spinal cord. Over 90%, if not all, SP positive DRG neurons also contain CGRP (Wiesenfeld-Hallin et al., 1984, Lawson, 1995).

CGRP and SP contribute to inflammation

CGRP and SP are synthesized in the cell body of the DRG neurons and transported to both peripheral and central terminals (Takahashi and Otsuka, 1975, Zochodne et al., 2001). Peripheral release of CGRP and SP contributes to neurogenic inflammation. Studies show that peripheral release of SP from the sensory nerve terminal increases plasma exudation and that CGRP acts on arterioles causing vasodilation and increased local blood flow (Holzer, 1998). After peripheral inflammation, there is a rapid increase in CGRP and SP mRNA level (Bulling et al., 2001) and CGRP and SP protein expression in DRG cell bodies. This is followed by increased CGRP and SP levels in peripheral and central nerve root/axons and peripheral terminals in the inflamed tissue during both acute and chronic phases of inflammation (Donnerer et al., 1992, Hanesch et al., 1993a, Hanesch et al., 1993b). At the central terminal, CGRP and SP coexist in large dense-core vesicles and, upon stimulation, are co-released with glutamate. CGRP and SP modulate basal glutamate efflux in rat spinal cord slices (Kangrga et al., 1990, Kangrga and Randic, 1990) and are involved in the production of inflammatory hyperalgesia by enhancing the responsiveness of glutamate receptors (Otsuka and Konishi, 1976, Okano et al., 1998, Keast and Stephensen, 2000, Sun et al., 2003).

Section summary: CGRP and SP are two neuropeptides/neuromodulators that are neuronal markers for nociceptive DRG neurons. 1) CGRP and SP are mainly expressed in small to medium sized DRG neurons that give rise to nociceptive neurons; 2) CGRP and SP are co-released with glutamate at central and peripheral terminals, contributing to inflammatory hyperalgesia.

IV. Glutamate release in peripheral inflammation

Glutamate-glutamine cycle in peripheral nervous system

Glutamate is the major excitatory neurotransmitter released in both central and peripheral sensory neurons (Hammerschlag and Weinreich, 1972, Johnson, 1972, Miller et al., 2011). The production and degradation of the neurotransmitter glutamate occurs in a "glutamate-glutamine cycle" that exists between the neurons and glial cells. Simply stated for the CNS, neurons produce glutamate from glutamine via glutaminase (GLS) and package it for release via vesicular glutamate transporters (VGLUT). Once released, astrocytes take up glutamate from the extracellular space via excitatory amino acid transporters (EAAT), thereby terminating the extracellular action of glutamate. Astrocytes convert glutamate to glutamine by glutamine synthetase (GS) and shuttle glutamine to neurons by Na^+-coupled neutral amino acid transporters (SNAT) (Mackenzie and Erickson, 2004, Albrecht et al., 2007).

In the peripheral nervous system, satellite cells and Schwann cells are the counterparts of astrocytes in the CNS. Satellite cells surrounding DRG neuronal cell bodies contain GS for conversion of glutamate to glutamine and SNAT 3,4 for transport out of the cell (Miller et al., 2002). All DRG neurons are glutamatergic, taking up glutamine via SNAT 1,2 and using GLS as the enzyme for the conversion of glutamine to glutamate (Miller et al., 1993, Miller et al., 2002). In DRG neurons, glutamate is packaged into synaptic vesicles by VGLUT's 1-3 (Brumovsky et al., 2007, Brumovsky et al., 2011, Malet et al., 2013). In the DRG and sciatic nerve, neurons, satellite cells and Schwann cells contain EAATs for extracellular glutamate uptake. DRG neurons express excitatory amino acid transporter 3 (EAAC1/EAAT3), while satellite cells and Schwann cells express glutamate-aspartate transporter (GLAST/EAAT1) and glutamate transporter 1 (GLT-1/EAAT2) (Tao et al., 2004, Carozzi et al., 2008). The presence of glutamate-glutamine cycle helps to 1) maintain the glutamate content in metabolic and neurotransmitter pools; and 2) remove the excessive glutamate in the extracellular

space, thus protecting the neurons from glutamate-mediated excitotoxicity of glutamate (McKenna, 2007).

Besides functioning as the major excitatory neurotransmitter, glutamate is also a non-essential amino acid that is important for cellular metabolism and biosynthesis of proteins and purines (Miller et al., 2002). The presence of cytoplasmic glutamate and GLS in non-neuronal tissue suggests that localization of glutamate or GLS is not sufficient to separate the neurotransmitter pool from the metabolic pool, and taht VGLUTs are responsible for this separation. Similar to the release of other neurotransmitters, the release of glutamate from synaptic vesicles is a cellular process using fast exocytosis mediated by Ca^{2+} influx. Ca^{2+} influx triggers docking and fusion of the synaptic vesicles with the presynaptic membrane in response to depolarization. VGLUTs are the key proteins that transport glutamate into the synaptic vesicle for synaptic release (Shigeri et al., 2004), so the presence of VGLUTs is considered as a specific marker of glutamatergic neurons. VGLUT1-3 proteins are present in spinal cord, primary afferent cell bodies, central processes and terminals in glabrous and hairy skin (Li et al., 2003, Brumovsky et al., 2007).

At peripheral and central terminals, studies at cellular and ultrastructural levels demonstrate the presence of ionotropic (AMPA, NMDA, kainate) and metabotropic glutamate receptors (mGluR) in DRG neurons, myelinated and unmyelinated sensory nerve fibers and free nerve terminals in the skin (Sato et al., 1993, Carlton et al., 1995, Juranek and Lembeck, 1996, Willcockson and Valtschanoff, 2008) and central terminals in the spinal cord (Furuyama et al., 1993, Carlton et al., 1998). Ionotropic glutamate receptors are liganded cation channels on the nociceptive nerve terminal. They mediate sodium and calcium ion influx, and facilitate the depolarization of the nerve terminal (Carlton et al., 2001).

The presence of glutaminase, VGLUTs and glutamate receptors in nociceptive nerve terminal indicates that glutamate can be: 1) synthesized by glutaminase, 2) packed into synaptic vesicles via VGLUTs locally at the nerve terminal, and 3) activates the nerve terminal by binding to glutamate receptors.

Glutamate is implicated in nociceptive transmission in normal and inflamed states

As mentioned earlier, glutamatergic primary afferents also carry an "efferent" function. Glutamate is packaged in synaptic vesicles via VGLUTs and released at peripheral and/or central terminals in response to natural or electrical stimuli, chemical activation, pro-inflammatory mediators and nerve injury (Wheeler et al., 1966, Kangrga et al., 1990, Kangrga and Randic, 1990, al-Ghoul et al., 1993, deGroot et al., 2000, Zahn et al., 2002, Miller et al., 2011, Inquimbert et al., 2012).

Subcutaneous injection of glutamate and other glutamatergic agonists for glutamate receptors produces local inflammatory responses including mechanical allodynia, thermal hyperalgesia, and edema (Zhou et al., 1996, Du et al., 2001). Exogenous glutamate binds to the EAARs at the nociceptive nerve terminal, triggers sodium and calcium influx, and activates the nociceptive nerve terminal. The activated nociceptive nerve terminal releases the vasoactive neuropeptides CGRP and SP, which increase vasodilation and vascular permeability, cause neurogenic inflammation, and produce peripheral sensitization (Juranek and Lembeck, 1996, Willcockson and Valtschanoff, 2008, Nakayama et al., 2010). The sensitization and edema produced by glutamate or other glutamatergic agonists can be attenuated by glutamate receptor antagonists in a dose-dependent manner (Du et al., 2006).

During inflammation, there are elevated glutamate levels and release at peripheral nerve terminals in the epidermis, synovial fluid in the knee joint, extracellular space and muscle (Lawand et al., 2000, Cairns et al., 2002, McNearney et al., 2004). The elevated release may lead to increased activation of glutamate receptors in peripheral tissue, especially at nociceptive nerve terminals (Carlton et al., 1995, Zhou et al., 1996, Lawand et al., 1997, Coggeshall and Carlton, 1998). At central terminals, elevated glutamate release in the spinal cord has been observed in response to peripheral tissue or nerve injury in studies using paw incision and nerve injury models (al-Ghoul et al., 1993, Zahn et al., 2002). Glutamate also co-localizes and co-releases with CGRP and SP at central terminals in the spinal cord (Merighi et al., 1991). These neuropeptides can augment glutamate release and contribute to the

strengthening of glutamatergic synapses, which is a potential mechanism for central sensitization (Kangrga et al., 1990, Kangrga and Randic, 1990, Okano et al., 1998).

Section summary: 1) Glutamate is the major neurotransmitter in peripheral and central sensory neurons; 2) Glutamate can function as a sensitizer by binding to glutamate receptors at peripheral nerve terminals. 3) Peripheral glutamate release, together with neuropeptides, contributes to neurogenic inflammation.

V. Glutaminase as a novel pharmacologic target for potential analgesic drugs

Phosphate-activated glutaminase (GLS, PAG) is an important enzyme in the glutamate-glutamine cycle. It has been generally accepted that there exist two isoforms of GLS. GLS utilizes glutamine as precursor and catalyzes glutamine and H_2O to glutamate and ammonia (Curthoys and Watford, 1995). Under normal conditions, GLS is synthesized in the neuronal cytoplasm and transported to the outer face of mitochondrial inner membrane where the major glutamate synthesis occurs.

The kidney and brain share the same GLS isoforms (kidney/brain GLS), and these isoforms have a wider distribution found in lung, small intestine and spinal cord. Liver GLS is found in the liver hepatocytes around the portal region and is different from the kidney/brain GLS (Curthoys and Watford, 1995, Kvamme et al., 2001).

Glutamatergic neurons also support *de novo* synthesis of glutamate from α-ketoglutarate from the TCA cycle via transamination using aspartate aminotransferase. However, at least 70% of glutamate in the neurotransmitter pool is recycled via the glutamate-glutamine cycle, involving brain/kidney GLS (Hamberger et al., 1979a, Hamberger et al., 1979b, Peng et al., 1993, Hertz et al., 1999, Hertz, 2004, Waagepetersen et al., 2005).

A brief review of GLS function and regulation in the central nervous system

The concentration of glutamate in synaptic vesicles is in millimolar concentrations (Nedergaard et al., 2002) while extracellular concentrations of glutamate in the synaptic cleft are in nanomolar ranges.

The low concentration in synaptic cleft is maintained by glial cell and presynaptic neuron uptake. Excess glutamate from the synaptic cleft must be removed after the completion of a signaling event to maintain its homeostatic levels. Excess glutamate is detrimental to neurons due to overstimulation causing excitotoxicity, which is implicated in several CNS neurodegenerative diseases (Lipton and Rosenberg, 1994). Existing strategies for disease treatment are aimed at suppressing glutamate neurotoxicity by enhancement of glial glutamate transportation or blockade of the postsynaptic glutamate receptors. The effectiveness of these strategies in clinical trials is limited by the disrupted physiological glutamate signaling in non-targeted areas of the central nervous system. In recent years glutaminase dysregulation has gained increased attention in CNS neurodegenerative diseases related to glutamate excitotoxicity, such as experimental cerebral ischemia, Alzheimer's disease (AD) and HIV-associated neurocognitive disease (HAND) (Burbaeva et al., 2005, Takeuchi et al., 2008, Potter et al., 2013, Ye et al., 2013).

Damaged cultured cortical neurons have an elevated mitochondrial glutaminase activity that contributes to the prolonged hydrolysis of extracellular glutamine to glutamate (Newcomb et al., 1997). Using an *in vivo* permanent focal ischemia model, it has been shown that activation of mitochondrial GLS occurs at the periphery of the ischemic site and accounts for the continued total elevation of extracellular glutamate in the ischemic tissue (Newcomb et al., 1998). One study, comparing the glutamate metabolizing enzymes in prefrontal cortex between controls and Alzheimer's disease patients, demonstrates that glutaminase is elevated in AD patients and the elevation of glutaminase may contribute to the elevated production of glutamate leading to excitotoxicity and neuronal death in the brain with AD (Burbaeva et al., 2005). Elevated glutaminase is also found in the neurons around the site of inflammation in a murine model of HIV-1 encephalitis (Ye et al., 2013). These studies provide evidence that, under pathological conditions, up-regulated neuronal GLS leads to increased production of glutamate leading to neuronal cell death and apoptosis, which may be the direct cause of neurotoxicity in neurodegenerative disease. It also suggests an

alternative therapeutic strategy in neurodegenerative disease for inhibition of glutaminase to suppress glutamate release and achieve neuroprotection (Hinoi et al., 2005, Potter et al., 2013).

Expression and elevation of GLS in DRG neurons during peripheral inflammation

In the peripheral nervous system, studies using immunohistochemistry and Western blots show that DRG neurons express the kidney isoform of GLS in neurons of all sizes, but no GLS expression occurs in satellite cells or Schwann cells (Miller et al., 1993, Miller et al., 2012). The percentage of GLS-expressing neurons in rodent DRG has varied between different laboratories, depending on the types of fixation and staining techniques used (Cangro et al., 1985, Battaglia and Rustioni, 1988). In our laboratory, an optimized fixative is used that has a low paraformaldehyde and high picric acid concentration. This better preserves the epitopes of the target protein and significantly increase the percentage of GLS positive neurons in DRGs. Using this fixative and other techniques for optimal immunohistochemistry, GLS is detected in all DRG neurons (Hoffman et al., 2010). This better matches the presence of other glutamatergic components in mechanoreceptor DRG neurons (Coggeshall and Carlton, 1998, Fagan and Cahusac, 2001). It also is in keeping with glutamate serving as the excitatory amino acid neurotransmitter for fast synaptic transmission.

As mentioned previously, there is excess production and release of glutamate during peripheral inflammation (Lawand et al., 2000, Du et al., 2006) and the inflammation-induced nociceptive behaviors can be suppressed by antagonizing glutamate receptors (Ren and Dubner, 1993). Current studies show that elevated glutamate is mainly released by peripheral nerve terminals, but what contribution GLS has to the elevated amount of glutamate has not been identified. In the glutamate-glutamine cycle, GLS is the key enzyme for establishing the neurotransmitter pool via converting glutamine to glutamate (Hertz, 2004), so it is reasonable to evaluate whether GLS alters during the course of inflammation.

Previous studies from our lab evaluated the alteration of GLS in rat DRG during peripheral inflammation. At 7 days of inflammation using rat adjuvant-induced arthritis (AIA) model, there is elevated GLS expression levels and enzymatic activity in DRG neurons, and increased levels of GLS

and glutamate also are observed in the skin in the peripheral terminals at the site of inflammation (Miller et al., 2012). An elevation of mitochondrial GLS, as well as cytosolic GLS is observed after 7 days of AIA (Miller et al., 2012) A temporal study evaluated the alteration of GLS at one, two, four and eight days during AIA and revealed a peak elevation of GLS at day four of AIA in all sizes of DRG neurons that persists in small neurons at eight days of inflammation (Hoffman, 2009).

DRG neurons are heterogeneous in cell body size and neurochemical markers. If the evaluation of the alteration is based on the total population, the presence of large DRG neurons will skew the data and mask the contribution of each potential subpopulation. By separating the data by DRG neuronal size, the major GLS alteration occurs in small DRG neurons that give rise to mainly C-fibers, most of which are nociceptors.

Potential axoplasmic transport of glutaminase after inflammation

Elevated GLS is found in DRG neuron soma and peripheral terminals after inflammation (Hoffman, 2009, Miller et al., 2012). Elevation of GLS levels in peripheral terminals suggests that it is transported from the cell body in the peripheral axons.

Many studies show that disrupted axonal transport results in accumulation of enzymes and organelles that can be observed at sides proximal and distal to the site of ligature/crush/chemical blockage (Zelena et al., 1968, McDougal et al., 1981). This simple method confirms the existence of axoplamic transport and provides a convenient method to study the machineries and mechanisms that are required for axonal transport, a crucial cellular process for development and function of neurons. Mitochondria are one of the organelles in motion in axoplasm and GLS activity is found accumulated at the proximal side of sciatic nerve ligature (McDougal et al., 1981). Studies on brain/kidney GLS show that there are two major sub-cellular locations of GLS, a cytoplasmic GLS and mitochondrial GLS. The mitochondrial GLS is considered active and the cytoplasmic GLS is inactive due to the high matrix concentration of the inhibitor glutamate (Kvamme et al., 2001, Miller et al., 2012). Studies using an *in vitro* culture system showed that mitochondria accumulate at the site of NGF stimulation by stimulating the axons with NGF-coated beads at points distant from their cell bodies or

growth cones (Chada and Hollenbeck, 2003, 2004). Although the transport machinery of glutaminase in the peripheral axons has not been identified, these studies support the hypothesis that elevated glutaminase can be mobilized and transported from DRG neuronal cell body to the peripheral nerve terminal for peripheral glutamate production.

GLS can be a novel target for analgesia

Drugs targeting glutamate receptors and glutamate transporters as treatment for excitotoxicity have side effects such as disrupting normal glutamate signaling in the central nervous system. All glutamate receptors participate in initiation, sensitization, modulation or maintenance of pain in different animal or human models, but with different temporal profiles and on different scales. Each receptor type differs in cellular distribution, affinity for glutamate and activation of downstream signaling pathways. Thus far, relatively limited studies have tried to decrease access to their common endogenous ligand, glutamate. The downstream signals produced by glutamate are a net effect of activation of different glutamate receptors and it is difficult to separate the attribution of each glutamate receptor. Increased glutamate production in central and peripheral terminals of primary afferents may come from excessive synthesis by elevated GLS at both terminals. As the neurotransmitter and sensitizer of the peripheral nerve terminal, suppressing glutamate synthesis by inhibition of glutaminase at the peripheral terminal may have an analgesic effect.

6-diazo-5-oxo-L-norleucine (DON) is a glutamine analog and shares similar uptake mechanisms as glutamine in neurons by the sodium-coupled neutral amino acid (System N/A) transporters (SNAT1, 2). DON irreversibly binds to the glutamine acceptor site in phosphate-activated glutaminase and interferes with glutamate synthesis. It previously has been used as a chemotherapeutic drug and evaluated in cancer research. DON also has been used as an effective GLS inhibitor to block glutamate production and release in the central nervous system. It has been shown that DON reduces the calcium-specific release of glutamate from synaptosomes in a concentration-dependent manner without affecting the calcium-specific release of aminobutyric acid (GABA), glycine or serotonin (Sherman and Mott, 1986). DON effectively abolishes glutamate immunoreactivity in cat

sensorimotor cortex via inhibition of GLS, providing further evidence that GLS is the major synthetic enzyme contributing to neurotransmitter pool (Conti and Minelli, 1994). GLS inhibition with DON also decreases glutamate release from activated microglia, rescues neuronal death in a dose-dependent manner in vitro, and attenuates clinical scores in ischemic brain injury and experimental autoimmune encephalomyelitis in mice (Takeuchi et al., 2008, Shijie et al., 2009).

DON has been administrated in animal models in our laboratory to study the effect of peripheral inhibition of GLS and glutamate release during inflammation. DON suppresses carrageenan-evoked c-fos immunoreactivity in the rat spinal cord 3 h after carrageenan-induced hindpaw inflammation (Hoffman and Miller, 2010). In the rat AIA model, DON attenuates thermal hyperalgesia and mechanical allodynia within 6 h after application at 4-7 d AIA (Miller, 2007). These results support the idea that peripheral inhibition of GLS may be a novel therapeutic target for analgesia.

Section summary: 1) Elevated GLS contributes to excess glutamate synthesis and release and the neurotoxicity of glutamate leads to neuronal death in the central nervous system; 2) GLS inhibition shows promising therapeutic effects on glutamate excitotoxicity via suppressing glutamate release in alleviating neurodegenerative diseases; 3) Elevated GLS also is found in primary sensory neurons and is related to increased nociceptive behavior after peripheral inflammation; 4) Peripheral inhibition of GLS can be an alternative strategy for pain relief.

VI. Adjuvant induced arthritis as an animal model for the study of pain.

In the current project, adjuvant induced arthritis (AIA) will be used as the animal model that mimics rheumatoid arthritis for the majority of the studies. This model is produced by a single unilateral subcutaneous injection of 150 µl of 1:1 of saline/CFA emulsion (complete Freund's adjuvant, 75 µg/150 µl) in the hindpaw plantar surface of adult Sprague-Dawley (SD) rats with mixed sexes. Complete Freund's adjuvant is a solution of inactive and dried *Mycobacterium tuberculosis* suspended in mineral oil (1mg/ml) (Wollweber, 1990). For subcutaneous injection, CFA is emulsified in a 1:1 solution with saline or phosphate buffered saline (PBS). Local injection of CFA in the

hindpaw produces a severe inflammation that characterized by erythema (redness), edema (swelling), hyperthermia (heat), hypersensitivity (pain) and loss of function (Stein et al., 1988, Fehrenbacher et al., 2012). Other than the above five hallmark signs of inflammation, animals with adjuvant-induced inflammation exhibit normal grooming and normal levels of activity but tend to limp and guard the inflamed paw (Stein et al., 1988, Fehrenbacher et al., 2012). The mechanisms of CFA-induced inflammation have not been not fully understood. Histological studies report that by 24 h, infiltration of neutrophils and macrophages are observed at the site of injection, and the accumulation is correlated with dose of the CFA (Harper et al., 2001). The hallmarks of inflammation in the CFA model are associated with neurogenic inflammation, which will be introduced in detail in next section. It is an established model that simulates various clinical phases of inflammation.

The dose of CFA, rat strain and sex were taken into consideration in the current project. Different doses of CFA have been found across the literature. The common dose is a single injection of 75-150 µl in rats (Galeazza et al., 1995, Nicholas et al., 1999, Miller et al., 2012) and 5-10 µl in mice (Gauldie et al., 2004, Scherrer et al., 2010). One study reports that a higher dose (250 µg *Mycobacterium tuberculosis* suspended in 50 µl paraffin oil) of adjuvant in the hindpaw produces contralateral inflammation and arthritis after 14 days, but without the complicating effects seen in adjuvant induced chronic polyarthritis induced by CFA injection at the base of the tail (Donaldson et al., 1993). Similar dose-dependent increase of tissue IL-1β levels and immune cell infiltration is observed when applied to the temporomandibular joint (Harper et al., 2001). The dose of CFA for the current study has been used extensively in our laboratory and produces a robust and reproducible inflammation. Rat strain/stock and sex differences also are taken into consideration. The major advantage of Sprague-Dawley rats is their relative calmness compared to other rat strains and their relative ease to handle. SD rats have more consistent immune and behavior responses compared to other laboratory rat strains (Zhang et al., 2003, Cai et al., 2006, Vukojevic et al., 2007). Gender difference is suggested in morphine potency and opioid receptor subtype expression level between

male and female rats (Cook and Nickerson, 2005, Wang et al., 2006). The SD rat shows the minimum susceptibility between genders on behavioral level in the AIA model (Bradshaw et al., 2000, DeLeo and Rutkowski, 2000, Faraday, 2002).

Naïve SD rats with no injection will be used as controls in the studies. Sterile saline is the most popular choice of vehicle control when using the AIA model. However, it has observed that sterile saline injection plus the needle wound produces a slight local, inflammation hypersensitivity in the behavior tests and alteration in protein production in the DRG (see Chapter II and IV) (Hoffman, 2009). These changes resolve within 2 d without other noticeable long-term behavioral alteration. It has been suggested that always including a non-treated animal group as naïve controls better reflects the progress of inflammation and effect of analgesic or anti-inflammatory drugs in experimental animals (Fehrenbacher et al., 2012). CFA is a suspension with killed bacteria and the emulsion of sterile saline and mineral oil without the bacteria is called "incomplete Freund's adjuvant." It also produces an acute inflammatory response, so it cannot be used as an appropriate control. In the current study, the major aim is to measure the alteration of comparisons between a naïve/normal animals and inflamed animals, instead of a minor inflammation versus a major inflammation. Thus, in current project, naïve/normal controls are used in all experiments, and vehicle control is only included in the experiments with pharmacological treatment (Chapter IV).

The single injection of CFA into the hindpaw producing a monoarthritis model is a preferred animal model in this study for the following reasons. First, the monoarthritis that develops in the rat inflamed hindpaw and the biochemical changes are localized to the ipsilateral lumbar dorsal root ganglion and lumbar dorsal horn. The rats do not develop the chronic polyarthritis of all limbs as observed following systemic injection of adjuvant. The unilateral, increased nociceptive sensitivity allows us to compare between the normal and sensitized region. Secondly, the local injection of Freund's adjuvant likely results in inflammation in both cutaneous tissue and deeper tissue to produce a more robust local inflammation affecting the functions of more (or additional or maximal number of) primary

afferent nerve terminals. Third, unilateral AIA produces a persistent inflammation that reaches a peak response within 6 h and lasts for several weeks, while other reagents e.g., formalin, carregeenan, capsaicin, nerve growth factor, produce a less robust and relatively short inflammation that resolves on its own within 24 h - 4 d (Ren and Dubner, 1999). The AIA model allows us to evaluate the alteration of several pro-inflammatory mediators during the chronic phase of the inflammation.

Section summary: The unilateral AIA model is an useful animal model for studying inflammation. It allows us: 1) observe nocifensive behavior for comparison with normal responses; 2) evaluate cellular and molecular alterations of target proteins or substances; 3) compare and evaluate the effects of pharmacologic treatment on pain and inflammation.

VII. Summary

Pain and temperature are sensed by a subset of DRG neurons. Calcitonin gene-related peptide (CGRP) and substance P (SP) are neuropeptides expressed in small- and medium-sized, nociceptive DRG neurons. A phenotypic switch occurs in DRG neurons when peripheral tissue is challenged by inflammation, leading to elevated expression of both sensory neuropeptides that is correlated with elevated nociceptive behavior under peripheral inflammation. The two neuropeptides are "classic" neurochemical markers of nociceptive neurons. It is known that during peripheral inflammation, GLS protein expression is elevated in DRG neurons, particularly small- and medium-sized, presumably nociceptive, neurons during the chronic phase of inflammation (Hoffman, 2009). It is of importance to evaluate the temporal response of glutaminase (GLS) expression level in the peptidergic subpopulation of DRG neurons after adjuvant-induced arthritis ***(Chapter II).***

It has been demonstrated that elevated GLS expression level in AIA is correlated with increased nocifensive behavior. Glutamate release increases at the peripheral terminal of the primary afferents during inflammation, and it is reasonable to assume that this is due to the amount of GLS in the peripheral terminals. The only source of GLS protein in the peripheral terminals is from the neuronal

cell bodies located in the DRG. It is reasonable to anticipate that if there is elevation of GLS in the DRG neuronal cell bodies, then elevated GLS can be observed in the peripheral nerve during the acute phase of AIA in accordance with the temporal alteration pattern found in neuronal cell bodies ***(Chapter III)***.

During inflammation, several inflammatory mediators will be co-released by sensory nerve terminals causing neurogenic inflammation. Those molecules act as sensitizers in augmenting nociception. Elevated glutamate is released from peripheral sensory terminals during inflammation. By binding to glutamate receptors on peripheral terminals of primary afferents, glutamate functions as a sensitizer contributing to peripheral sensitization in addition to its role as a major neurotransmitter. This has been demonstrated by pharmacological studies using exogenous glutamate receptor ligands and antagonists or evaluating glutamate concentration at the target tissue during inflammation. Limited studies have been performed to evaluate the effect of reducing glutamate release by inhibiting GLS, its synthetic enzyme, in the study of pain. DON is an effective glutamine antagonist that inhibits GLS activity and glutamate synthesis. In the AIA model, the administration of DON has effectively reversed hyperalgesia and allodynia at 4-7 d AIA. The administration of DON occurred at 3 d post-inflammation when glutamate has elevated due to increased GLS concentrations in the peripheral terminals. In another study using the carrageenan-induced inflammation model, depletion of peripheral glutamate by pre- and concurrent administration of DON before the initiation of inflammation suppresses paw edema in the inflamed paw and c-fos immunoreactivity in the spinal cord dorsal horn during the acute phase of inflammation. It will be necessary to evaluate the effect of pre-depletion of glutamate on alteration of nociceptive behavior in both acute and chronic phases of AIA. It is also necessary to find out if pre-depletion of glutamate can modify the phenotypic switch in the DRG neuronal cell body during the chronic inflammation ***(Chapter IV)***.

VIII. List of hypotheses/aims

Aim#1 To determine and characterize the temporal response of glutaminase levels in peptidergic DRG neuronal cell bodies during acute and chronic phases in rat adjuvant-induced arthritis model.

Aim#2 If elevated glutaminase production occurs in DRG neuronal cell bodies in response to inflammation and if there is elevated glutaminase found in the hindpaw, we hypothesize that altered glutaminase levels could be detected in the sciatic nerve at specific time points during AIA.

Aim#3 Considering the role of glutamate as a sensitizer to the peripheral nerve terminal on the generation, modulation, and maintenance of peripheral sensitization at different time points during inflammation, we propose that blocking the production of glutamate in the peripheral target tissues prior to inflammation may 1) alleviate the nociceptive behaviors; 2) inhibit the alteration of neurochemical characteristics during peripheral inflammation in the peptidergic DRG neuronal cell bodies.

References

al-Ghoul WM, Volsi GL, Weinberg RJ, Rustioni A (1993) Glutamate immunocytochemistry in the dorsal horn after injury or stimulation of the sciatic nerve of rats. Brain research bulletin 30:453-459.

Albers KM, Davis BM (2007) The skin as a neurotrophic organ. The Neuroscientist : a review journal bringing neurobiology, neurology and psychiatry 13:371-382.

Albrecht J, Sonnewald U, Waagepetersen HS, Schousboe A (2007) Glutamine in the central nervous system: function and dysfunction. Frontiers in bioscience: a journal and virtual library 12:332-343.

Amann R, Schuligoi R, Herzeg G, Donnerer J (1996) Intraplantar injection of nerve growth factor into the rat hind paw: local edema and effects on thermal nociceptive threshold. Pain 64:323-329.

Battaglia G, Rustioni A (1988) Coexistence of glutamate and substance P in dorsal root ganglion neurons of the rat and monkey. The Journal of comparative neurology 277:302-312.

Bennett G, al-Rashed S, Hoult JR, Brain SD (1998) Nerve growth factor induced hyperalgesia in the rat hind paw is dependent on circulating neutrophils. Pain 77:315-322.

Bradshaw H, Miller J, Ling Q, Malsnee K, Ruda MA (2000) Sex differences and phases of the estrous cycle alter the response of spinal cord dynorphin neurons to peripheral inflammation and hyperalgesia. Pain 85:93-99.

Brumovsky P, Watanabe M, Hokfelt T (2007) Expression of the vesicular glutamate transporters-1 and -2 in adult mouse dorsal root ganglia and spinal cord and their regulation by nerve injury. Neuroscience 147:469-490.

Brumovsky PR, Robinson DR, La JH, Seroogy KB, Lundgren KH, Albers KM, Kiyatkin ME, Seal RP, Edwards RH, Watanabe M, Hokfelt T, Gebhart GF (2011) Expression of vesicular glutamate transporters type 1 and 2 in sensory and autonomic neurons innervating the mouse colorectum. The Journal of comparative neurology 519:3346-3366.

Bulling DG, Kelly D, Bond S, McQueen DS, Seckl JR (2001) Adjuvant-induced joint inflammation causes very rapid transcription of beta-preprotachykinin and alpha-CGRP genes in innervating sensory ganglia. Journal of neurochemistry 77:372-382.

Burbaeva G, Boksha IS, Tereshkina EB, Savushkina OK, Starodubtseva LI, Turishcheva MS (2005) Glutamate metabolizing enzymes in prefrontal cortex of Alzheimer's disease patients. Neurochemical research 30:1443-1451.

Cai X, Wong YF, Zhou H, Xie Y, Liu ZQ, Jiang ZH, Bian ZX, Xu HX, Liu L (2006) The comparative study of Sprague-Dawley and Lewis rats in adjuvant-induced arthritis. Naunyn-Schmiedeberg's archives of pharmacology 373:140-147.

Cairns BE, Gambarota G, Svensson P, Arendt-Nielsen L, Berde CB (2002) Glutamate-induced sensitization of rat masseter muscle fibers. Neuroscience 109:389-399.

Campenot RB, MacInnis BL (2004) Retrograde transport of neurotrophins: fact and function. Journal of neurobiology 58:217-229.

Cangro CB, Sweetnam PM, Wrathall JR, Haser WB, Curthoys NP, Neale JH (1985) Localization of elevated glutaminase immunoreactivity in small DRG neurons. Brain research 336:158-161.

Carlton SM, Hargett GL (2002) Stereological analysis of Ca(2+)/calmodulin-dependent protein kinase II alpha -containing dorsal root ganglion neurons in the rat: colocalization with isolectin Griffonia simplicifolia, calcitonin gene-related peptide, or vanilloid receptor 1. The Journal of comparative neurology 448:102-110.

Carlton SM, Hargett GL, Coggeshall RE (1995) Localization and activation of glutamate receptors in unmyelinated axons of rat glabrous skin. Neuroscience letters 197:25-28.

Carozzi VA, Canta A, Oggioni N, Ceresa C, Marmiroli P, Konvalinka J, Zoia C, Bossi M, Ferrarese C, Tredici G, Cavaletti G (2008) Expression and distribution of 'high affinity' glutamate transporters GLT1, GLAST, EAAC1 and of GCPII in the rat peripheral nervous system. Journal of anatomy 213:539-546.

Chada SR, Hollenbeck PJ (2003) Mitochondrial movement and positioning in axons: the role of growth factor signaling. The Journal of experimental biology 206:1985-1992.

Chada SR, Hollenbeck PJ (2004) Nerve growth factor signaling regulates motility and docking of axonal mitochondria. Current biology : CB 14:1272-1276.

Chiu IM, von Hehn CA, Woolf CJ (2012) Neurogenic inflammation and the peripheral nervous system in host defense and immunopathology. Nature neuroscience 15:1063-1067.

Coggeshall RE, Carlton SM (1998) Ultrastructural analysis of NMDA, AMPA, and kainate receptors on unmyelinated and myelinated axons in the periphery. The Journal of comparative neurology 391:78-86.

Conti F, Minelli A (1994) Glutamate immunoreactivity in rat cerebral cortex is reversibly abolished by 6-diazo-5-oxo-L-norleucine (DON), an inhibitor of phosphate-activated glutaminase. J Histochem Cytochem 42:717-726.

Cook CD, Nickerson MD (2005) Nociceptive sensitivity and opioid antinociception and antihyperalgesia in Freund's adjuvant-induced arthritic male and female rats. The Journal of pharmacology and experimental therapeutics 313:449-459.

Curthoys NP, Watford M (1995) Regulation of glutaminase activity and glutamine metabolism. Annual review of nutrition 15:133-159.

Dai Y, Fukuoka T, Wang H, Yamanaka H, Obata K, Tokunaga A, Noguchi K (2004) Contribution of sensitized P2X receptors in inflamed tissue to the mechanical hypersensitivity revealed by phosphorylated ERK in DRG neurons. Pain 108:258-266.

deGroot J, Zhou S, Carlton SM (2000) Peripheral glutamate release in the hindpaw following low and high intensity sciatic stimulation. Neuroreport 11:497-502.

DeLeo JA, Rutkowski MD (2000) Gender differences in rat neuropathic pain sensitivity is dependent on strain. Neuroscience letters 282:197-199.
Djouhri L, Dawbarn D, Robertson A, Newton R, Lawson SN (2001) Time course and nerve growth factor dependence of inflammation-induced alterations in electrophysiological membrane properties in nociceptive primary afferent neurons. The Journal of neuroscience : the official journal of the Society for Neuroscience 21:8722-8733.
Djouhri L, Lawson SN (1999) Changes in somatic action potential shape in guinea-pig nociceptive primary afferent neurones during inflammation in vivo. The Journal of physiology 520 Pt 2:565-576.
Djouhri L, Lawson SN (2004) Abeta-fiber nociceptive primary afferent neurons: a review of incidence and properties in relation to other afferent A-fiber neurons in mammals. Brain research Brain research reviews 46:131-145.
Donaldson LF, McQueen DS, Seckl JR (1994) Local anaesthesia prevents acute inflammatory changes in neuropeptide messenger RNA expression in rat dorsal root ganglia neurons. Neuroscience letters 175:111-113.
Donaldson LF, Seckl JR, McQueen DS (1993) A discrete adjuvant-induced monoarthritis in the rat: effects of adjuvant dose. J Neurosci Methods 49:5-10.
Donnerer J, Schuligoi R, Stein C (1992) Increased content and transport of substance P and calcitonin gene-related peptide in sensory nerves innervating inflamed tissue: evidence for a regulatory function of nerve growth factor in vivo. Neuroscience 49:693-698.
Du J, Zhou S, Carlton SM (2006) Kainate-induced excitation and sensitization of nociceptors in normal and inflamed rat glabrous skin. Neuroscience 137:999-1013.
Ehlers MD, Kaplan DR, Price DL, Koliatsos VE (1995) NGF-stimulated retrograde transport of trkA in the mammalian nervous system. The Journal of cell biology 130:149-156.
Fagan BM, Cahusac PM (2001) Evidence for glutamate receptor mediated transmission at mechanoreceptors in the skin. Neuroreport 12:341-347.
Faraday MM (2002) Rat sex and strain differences in responses to stress. Physiology & behavior 75:507-522.
Fehrenbacher JC, Vasko MR, Duarte DB (2012) Models of inflammation: Carrageenan- or complete Freund's Adjuvant (CFA)-induced edema and hypersensitivity in the rat. Current protocols in pharmacology / editorial board, SJ Enna Chapter 5:Unit5 4.
Fullmer JM, Riedl MS, Higgins L, Elde R (2004) Identification of some lectin IB4 binding proteins in rat dorsal root ganglia. Neuroreport 15:1705-1709.
Galeazza MT, Garry MG, Yost HJ, Strait KA, Hargreaves KM, Seybold VS (1995) Plasticity in the synthesis and storage of substance P and calcitonin gene-related peptide in primary afferent neurons during peripheral inflammation. Neuroscience 66:443-458.
Gauldie SD, McQueen DS, Clarke CJ, Chessell IP (2004) A robust model of adjuvant-induced chronic unilateral arthritis in two mouse strains. J Neurosci Methods 139:281-291.
Hamberger A, Chiang GH, Sandoval E, Cotman CW (1979a) Glutamate as a CNS transmitter. II. Regulation of synthesis in the releasable pool. Brain research 168:531-541.
Hamberger AC, Chiang GH, Nylen ES, Scheff SW, Cotman CW (1979b) Glutamate as a CNS transmitter. I. Evaluation of glucose and glutamine as precursors for the synthesis of preferentially released glutamate. Brain research 168:513-530.
Hammerschlag R, Weinreich D (1972) Glutamic acid and primary afferent transmission. Advances in biochemical psychopharmacology 6:165-180.
Hanesch U, Pfrommer U, Grubb BD, Heppelmann B, Schaible HG (1993a) The proportion of CGRP-immunoreactive and SP-mRNA containing dorsal root ganglion cells is increased by a unilateral inflammation of the ankle joint of the rat. Regul Pept 46:202-203.
Hanesch U, Pfrommer U, Grubb BD, Schaible HG (1993b) Acute and chronic phases of unilateral inflammation in rat's ankle are associated with an increase in the proportion of calcitonin

gene-related peptide-immunoreactive dorsal root ganglion cells. The European journal of neuroscience 5:154-161.
Harper RP, Kerins CA, McIntosh JE, Spears R, Bellinger LL (2001) Modulation of the inflammatory response in the rat TMJ with increasing doses of complete Freund's adjuvant. Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society 9:619-624.
Harrington AW, Ginty DD (2013) Long-distance retrograde neurotrophic factor signalling in neurons. Nature reviews Neuroscience 14:177-187.
Hertz L (2004) Intercellular metabolic compartmentation in the brain: past, present and future. Neurochemistry international 45:285-296.
Hertz L, Dringen R, Schousboe A, Robinson SR (1999) Astrocytes: glutamate producers for neurons. Journal of neuroscience research 57:417-428.
Hinoi E, Takarada T, Tsuchihashi Y, Yoneda Y (2005) Glutamate transporters as drug targets. Current drug targets CNS and neurological disorders 4:211-220.
Hoffman EM (2009) The role of dorsal root ganglion glutaminase in nociception - A novel therapeutic target for inflammatory pain. In: Ann Arbor: ProQuest/UMI.
Hoffman EM, Miller KE (2010) Peripheral inhibition of glutaminase reduces carrageenan-induced Fos expression in the superficial dorsal horn of the rat. Neuroscience letters 472:157-160.
Hoffman EM, Schechter R, Miller KE (2010) Fixative composition alters distributions of immunoreactivity for glutaminase and two markers of nociceptive neurons, Nav1.8 and TRPV1, in the rat dorsal root ganglion. J Histochem Cytochem 58:329-344.
Hoffman EM, Zhang Z, Anderson MB, Schechter R, Miller KE (2011) Potential mechanisms for hypoalgesia induced by anti-nerve growth factor immunoglobulin are identified using autoimmune nerve growth factor deprivation. Neuroscience 193:452-465.
Holzer P (1998) Neurogenic vasodilatation and plasma leakage in the skin. General pharmacology 30:5-11.
IASP (1986) Classification of chronic pain. Descriptions of chronic pain syndromes and definitions of pain terms. Prepared by the International Association for the Study of Pain, Subcommittee on Taxonomy. Pain Suppl 3:S1-226.
Inquimbert P, Bartels K, Babaniyi OB, Barrett LB, Tegeder I, Scholz J (2012) Peripheral nerve injury produces a sustained shift in the balance between glutamate release and uptake in the dorsal horn of the spinal cord. Pain 153:2422-2431.
Ji RR, Kohno T, Moore KA, Woolf CJ (2003) Central sensitization and LTP: do pain and memory share similar mechanisms? Trends Neurosci 26:696-705.
Johnson JL (1972) Glutamic acid as a synaptic transmitter in the nervous system. A review. Brain research 37:1-19.
Julius D, Basbaum AI (2001) Molecular mechanisms of nociception. Nature 413:203-210.
Juranek I, Lembeck F (1996) Evidence for the participation of glutamate in reflexes involving afferent, substance P-containing nerve fibres in the rat. Br J Pharmacol 117:71-78.
Kangrga I, Larew JS, Randic M (1990) The effects of substance P and calcitonin gene-related peptide on the efflux of endogenous glutamate and aspartate from the rat spinal dorsal horn in vitro. Neuroscience letters 108:155-160.
Kangrga I, Randic M (1990) Tachykinins and calcitonin gene-related peptide enhance release of endogenous glutamate and aspartate from the rat spinal dorsal horn slice. The Journal of neuroscience : the official journal of the Society for Neuroscience 10:2026-2038.
Keast JR, Stephensen TM (2000) Glutamate and aspartate immunoreactivity in dorsal root ganglion cells supplying visceral and somatic targets and evidence for peripheral axonal transport. The Journal of comparative neurology 424:577-587.
Kvamme E, Torgner IA, Roberg B (2001) Kinetics and localization of brain phosphate activated glutaminase. Journal of neuroscience research 66:951-958.
Latremoliere A, Woolf CJ (2009) Central sensitization: a generator of pain hypersensitivity by central neural plasticity. J Pain 10:895-926.

Lawand NB, McNearney T, Westlund KN (2000) Amino acid release into the knee joint: key role in nociception and inflammation. Pain 86:69-74.
Lawand NB, Willis WD, Westlund KN (1997) Excitatory amino acid receptor involvement in peripheral nociceptive transmission in rats. European journal of pharmacology 324:169-177.
Lawson SN (1995) Neuropeptides in morphologically and functionally identified primary afferent neurons in dorsal root ganglia: substance P, CGRP and somatostatin. Progress in brain research 104:161-173.
Lawson SN (2002) Phenotype and function of somatic primary afferent nociceptive neurones with C-, Adelta- or Aalpha/beta-fibres. Exp Physiol 87:239-244.
Lawson SN, Crepps BA, Perl ER (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.
Lee KH, Chung K, Chung JM, Coggeshall RE (1986) Correlation of cell body size, axon size, and signal conduction velocity for individually labelled dorsal root ganglion cells in the cat. The Journal of comparative neurology 243:335-346.
Lewin GR, Ritter AM, Mendell LM (1993) Nerve growth factor-induced hyperalgesia in the neonatal and adult rat. The Journal of neuroscience : the official journal of the Society for Neuroscience 13:2136-2148.
Li JL, Fujiyama F, Kaneko T, Mizuno N (2003) Expression of vesicular glutamate transporters, VGluT1 and VGluT2, in axon terminals of nociceptive primary afferent fibers in the superficial layers of the medullary and spinal dorsal horns of the rat. The Journal of comparative neurology 457:236-249.
Lindsay RM, Harmar AJ (1989) Nerve growth factor regulates expression of neuropeptide genes in adult sensory neurons. Nature 337:362-364.
Lipton SA, Rosenberg PA (1994) Excitatory amino acids as a final common pathway for neurologic disorders. The New England journal of medicine 330:613-622.
Loeser JD, Treede RD (2008) The Kyoto protocol of IASP Basic Pain Terminology. Pain 137:473-477.
Lumpkin EA, Marshall KL, Nelson AM (2010) The cell biology of touch. The Journal of cell biology 191:237-248.
Mackenzie B, Erickson JD (2004) Sodium-coupled neutral amino acid (System N/A) transporters of the SLC38 gene family. Pflugers Archiv : European journal of physiology 447:784-795.
Malet M, Vieytes CA, Lundgren KH, Seal RP, Tomasella E, Seroogy KB, Hokfelt T, Gebhart GF, Brumovsky PR (2013) Transcript expression of vesicular glutamate transporters in lumbar dorsal root ganglia and the spinal cord of mice - Effects of peripheral axotomy or hindpaw inflammation. Neuroscience 248C:95-111.
Martin J (2012) Neuroanatomy Text and Atlas, Fourth Edition: Mcgraw-hill.
McDougal DB, Jr., Yu MJ, Gorin PD, Johnson EM, Jr. (1981) Transported enzymes in sciatic nerve and sensory ganglia of rats exposed to maternal antibodies against nerve growth factor. Journal of neurochemistry 36:1847-1852.
McKenna MC (2007) The glutamate-glutamine cycle is not stoichiometric: fates of glutamate in brain. Journal of neuroscience research 85:3347-3358.
McNearney T, Baethge BA, Cao S, Alam R, Lisse JR, Westlund KN (2004) Excitatory amino acids, TNF-alpha, and chemokine levels in synovial fluids of patients with active arthropathies. Clinical and experimental immunology 137:621-627.
Merighi A, Polak JM, Theodosis DT (1991) Ultrastructural visualization of glutamate and aspartate immunoreactivities in the rat dorsal horn, with special reference to the co-localization of glutamate, substance P and calcitonin-gene related peptide. Neuroscience 40:67-80.
Miller KE (2007) Method of alleviating chronic pain via peripheral glutaminase regulation. vol. 7,288,246 B2 United States: The Board of Regents of the University of Oklahoma.

Miller KE, Balbas JC, Benton RL, Lam TS, Edwards KM, Kriebel RM, Schechter R (2012) Glutaminase immunoreactivity and enzyme activity is increased in the rat dorsal root ganglion following peripheral inflammation. Pain Res Treat 2012:414697.
Miller KE, Douglas VD, Kaneko T (1993) Glutaminase immunoreactive neurons in the rat dorsal root ganglion contain calcitonin gene-related peptide (CGRP). Neuroscience letters 160:113-116.
Miller KE, Hoffman EM, Sutharshan M, Schechter R (2011) Glutamate pharmacology and metabolism in peripheral primary afferents: physiological and pathophysiological mechanisms. Pharmacol Ther 130:283-309.
Miller KE, Richards BA, Kriebel RM (2002) Glutamine-, glutamine synthetase-, glutamate dehydrogenase- and pyruvate carboxylase-immunoreactivities in the rat dorsal root ganglion and peripheral nerve. Brain research 945:202-211.
Molliver DC, Wright DE, Leitner ML, Parsadanian AS, Doster K, Wen D, Yan Q, Snider WD (1997) IB4-binding DRG neurons switch from NGF to GDNF dependence in early postnatal life. Neuron 19:849-861.
Nakayama T, Naono R, Ikeda T, Nishimori T (2010) NMDA and AMPA receptors contribute to the maintenance of substance P-induced thermal hyperalgesia. Neurosci Res 67:18-24.
Nedergaard M, Takano T, Hansen AJ (2002) Beyond the role of glutamate as a neurotransmitter. Nature reviews Neuroscience 3:748-755.
Neumann S, Doubell TP, Leslie T, Woolf CJ (1996) Inflammatory pain hypersensitivity mediated by phenotypic switch in myelinated primary sensory neurons. Nature 384:360-364.
Newcomb R, Pierce AR, Kano T, Meng W, Bosque-Hamilton P, Taylor L, Curthoys N, Lo EH (1998) Characterization of mitochondrial glutaminase and amino acids at prolonged times after experimental focal cerebral ischemia. Brain research 813:103-111.
Newcomb R, Sun X, Taylor L, Curthoys N, Giffard RG (1997) Increased production of extracellular glutamate by the mitochondrial glutaminase following neuronal death. The Journal of biological chemistry 272:11276-11282.
Nicholas RS, Winter J, Wren P, Bergmann R, Woolf CJ (1999) Peripheral inflammation increases the capsaicin sensitivity of dorsal root ganglion neurons in a nerve growth factor-dependent manner. Neuroscience 91:1425-1433.
Okano K, Kuraishi Y, Satoh M (1998) Involvement of spinal substance P and excitatory amino acids in inflammatory hyperalgesia in rats. Japanese journal of pharmacology 76:15-22.
Otsuka M, Konishi S (1976) Substance P and excitatory transmitter of primary sensory neurons. Cold Spring Harbor symposia on quantitative biology 40:135-143.
Otten U, Goedert M, Mayer N, Lembeck F (1980) Requirement of nerve growth factor for development of substance P-containing sensory neurones. Nature 287:158-159.
Peng L, Hertz L, Huang R, Sonnewald U, Petersen SB, Westergaard N, Larsson O, Schousboe A (1993) Utilization of glutamine and of TCA cycle constituents as precursors for transmitter glutamate and GABA. Developmental neuroscience 15:367-377.
Potter MC, Figuera-Losada M, Rojas C, Slusher BS (2013) Targeting the Glutamatergic System for the Treatment of HIV-Associated Neurocognitive Disorders. Journal of neuroimmune pharmacology : the official journal of the Society on NeuroImmune Pharmacology.
Price TJ, Flores CM (2007) Critical evaluation of the colocalization between calcitonin gene-related peptide, substance P, transient receptor potential vanilloid subfamily type 1 immunoreactivities, and isolectin B4 binding in primary afferent neurons of the rat and mouse. J Pain 8:263-272.
Priestley JV, Michael GJ, Averill S, Liu M, Willmott N (2002) Regulation of nociceptive neurons by nerve growth factor and glial cell line derived neurotrophic factor. Canadian journal of physiology and pharmacology 80:495-505.
Puehler W, Zollner C, Brack A, Shaqura MA, Krause H, Schafer M, Stein C (2004) Rapid upregulation of mu opioid receptor mRNA in dorsal root ganglia in response to peripheral inflammation depends on neuronal conduction. Neuroscience 129:473-479.

Ren K, Dubner R (1993) NMDA receptor antagonists attenuate mechanical hyperalgesia in rats with unilateral inflammation of the hindpaw. Neuroscience letters 163:22-26.
Ren K, Dubner R (1999) Inflammatory Models of Pain and Hyperalgesia. ILAR journal / National Research Council, Institute of Laboratory Animal Resources 40:111-118.
Richardson JD, Vasko MR (2002) Cellular mechanisms of neurogenic inflammation. The Journal of pharmacology and experimental therapeutics 302:839-845.
Scherrer G, Low SA, Wang X, Zhang J, Yamanaka H, Urban R, Solorzano C, Harper B, Hnasko TS, Edwards RH, Basbaum AI (2010) VGLUT2 expression in primary afferent neurons is essential for normal acute pain and injury-induced heat hypersensitivity. Proc Natl Acad Sci U S A 107:22296-22301.
Sherman AD, Mott J (1986) Effects of glutaminase inhibition on release of endogenous glutamic acid. Neuropharmacology 25:1353-1357.
Shigeri Y, Seal RP, Shimamoto K (2004) Molecular pharmacology of glutamate transporters, EAATs and VGLUTs. Brain research Brain research reviews 45:250-265.
Shijie J, Takeuchi H, Yawata I, Harada Y, Sonobe Y, Doi Y, Liang J, Hua L, Yasuoka S, Zhou Y, Noda M, Kawanokuchi J, Mizuno T, Suzumura A (2009) Blockade of glutamate release from microglia attenuates experimental autoimmune encephalomyelitis in mice. The Tohoku journal of experimental medicine 217:87-92.
Snider WD, McMahon SB (1998) Tackling pain at the source: new ideas about nociceptors. Neuron 20:629-632.
Stein C, Millan MJ, Herz A (1988) Unilateral inflammation of the hindpaw in rats as a model of prolonged noxious stimulation: alterations in behavior and nociceptive thresholds. Pharmacol Biochem Behav 31:445-451.
Stoeckel K, Schwab M, Thoenen H (1975) Specificity of retrograde transport of nerve growth factor (NGF) in sensory neurons: a biochemical and morphological study. Brain research 89:1-14.
Sun RQ, Lawand NB, Willis WD (2003) The role of calcitonin gene-related peptide (CGRP) in the generation and maintenance of mechanical allodynia and hyperalgesia in rats after intradermal injection of capsaicin. Pain 104:201-208.
Takahashi T, Otsuka M (1975) Regional distribution of substance P in the spinal cord and nerve roots of the cat and the effect of dorsal root section. Brain research 87:1-11.
Takeuchi H, Jin S, Suzuki H, Doi Y, Liang J, Kawanokuchi J, Mizuno T, Sawada M, Suzumura A (2008) Blockade of microglial glutamate release protects against ischemic brain injury. Experimental neurology 214:144-146.
Tao F, Liaw WJ, Zhang B, Yaster M, Rothstein JD, Johns RA, Tao YX (2004) Evidence of neuronal excitatory amino acid carrier 1 expression in rat dorsal root ganglion neurons and their central terminals. Neuroscience 123:1045-1051.
Verge VM, Richardson PM, Wiesenfeld-Hallin Z, Hokfelt T (1995) Differential influence of nerve growth factor on neuropeptide expression in vivo: a novel role in peptide suppression in adult sensory neurons. The Journal of neuroscience : the official journal of the Society for Neuroscience 15:2081-2096.
Vervest AC, Schimmel GH (1988) Taxonomy of pain of the IASP. Pain 34:318-321.
Vukojevic K, Lovric-Kojundzic S, Sapunar D (2007) Hyperalgesia-type response reveals no difference in pain-related behavior between Wistar and Sprague-Dawley rats. Bosnian journal of basic medical sciences / Udruzenje basicnih mediciniskih znanosti = Association of Basic Medical Sciences 7:121-126.
Waagepetersen HS, Qu H, Sonnewald U, Shimamoto K, Schousboe A (2005) Role of glutamine and neuronal glutamate uptake in glutamate homeostasis and synthesis during vesicular release in cultured glutamatergic neurons. Neurochemistry international 47:92-102.
Wang X, Traub RJ, Murphy AZ (2006) Persistent pain model reveals sex difference in morphine potency. American journal of physiology Regulatory, integrative and comparative physiology 291:R300-306.

Wheeler DD, Boyarsky LL, Brooks WH (1966) The release of amino acids from nerve during stimulation. Journal of cellular physiology 67:141-147.
Wiesenfeld-Hallin Z, Hokfelt T, Lundberg JM, Forssmann WG, Reinecke M, Tschopp FA, Fischer JA (1984) Immunoreactive calcitonin gene-related peptide and substance P coexist in sensory neurons to the spinal cord and interact in spinal behavioral responses of the rat. Neuroscience letters 52:199-204.
Willcockson H, Valtschanoff J (2008) AMPA and NMDA glutamate receptors are found in both peptidergic and non-peptidergic primary afferent neurons in the rat. Cell and tissue research 334:17-23.
Wollweber L (1990) E. Harlow and D. Lane (Editors), Antibodies: A Laboratory Manual. XIII + 726 S., 50 Abb., 62 Tab. Cold Spring Harbor 1988. Cold Spring Harbor Laboratory. $50.00. ISBN: 0-87969-314-2. Journal of Basic Microbiology 30:164-164.
Woolf CJ (2010) What is this thing called pain? The Journal of clinical investigation 120:3742-3744.
Woolf CJ, Doubell TP (1994) The pathophysiology of chronic pain--increased sensitivity to low threshold A beta-fibre inputs. Current opinion in neurobiology 4:525-534.
Woolf CJ, Ma Q (2007) Nociceptors--noxious stimulus detectors. Neuron 55:353-364.
Woolf CJ, Safieh-Garabedian B, Ma QP, Crilly P, Winter J (1994) Nerve growth factor contributes to the generation of inflammatory sensory hypersensitivity. Neuroscience 62:327-331.
Xu GY, Huang LY, Zhao ZQ (2000) Activation of silent mechanoreceptive cat C and Adelta sensory neurons and their substance P expression following peripheral inflammation. The Journal of physiology 528 Pt 2:339-348.
Ye L, Huang Y, Zhao L, Li Y, Sun L, Zhou Y, Qian G, Zheng JC (2013) IL-1beta and TNF-alpha induce neurotoxicity through glutamate production: a potential role for neuronal glutaminase. Journal of neurochemistry.
Yoshida S, Matsuda Y (1979) Studies on sensory neurons of the mouse with intracellular-recording and horseradish peroxidase-injection techniques. Journal of neurophysiology 42:1134-1145.
Zahn PK, Sluka KA, Brennan TJ (2002) Excitatory amino acid release in the spinal cord caused by plantar incision in the rat. Pain 100:65-76.
Zelena J, Lubinska L, Gutmann E (1968) Accumulation of organelles at the ends of interrupted axons. Z Zellforsch Mikrosk Anat 91:200-219.
Zhang RX, Lao L, Qiao JT, Ruda MA (2003) Strain differences in pain sensitivity and expression of preprodynorphin mRNA in rats following peripheral inflammation. Neuroscience letters 353:213-216.
Zhang X, Ju G, Elde R, Hokfelt T (1993) Effect of peripheral nerve cut on neuropeptides in dorsal root ganglia and the spinal cord of monkey with special reference to galanin. Journal of neurocytology 22:342-381.
Zhou S, Bonasera L, Carlton SM (1996) Peripheral administration of NMDA, AMPA or KA results in pain behaviors in rats. Neuroreport 7:895-900.
Zochodne DW, Cheng C, Miampamba M, Hargreaves K, Sharkey KA (2001) Peptide accumulations in proximal endbulbs of transected axons. Brain research 902:40-50.

CHAPTER II

TEMPORAL RESPONSE OF GLUTAMINASE IN CALCITONIN GENE-RELATED PEPTIDE- AND SUBSTANCE P- CONTAINING NEURONS IN THE RAT DORSAL ROOT GANGLION DURING ADJUVANT INDUCED ARTHRITIS

Abstract

Glutamate is the neurotransmitter utilized in primary sensory neurons of rat dorsal root ganglia (DRG). Glutaminase (GLS) is the synthetic enzyme that converts glutamine to glutamate. It has been reported in the previous study that the elevation of GLS-immunoreactivity (-ir) in DRG neuronal cell bodies during chronic peripheral inflammation. In the present study, we co-labeled GLS with two neurochemical markers for subpopulations of nociceptive DRG neurons, calcitonin gene-related peptide (CGRP) and substance P (SP). The temporal alteration of GLS-ir was assessed in the peptide-containing populations of neurons in response to unilateral adjuvant-induced arthritis (AIA). Ipsilateral 4th lumbar DRGs were collected and processed for immunohistochemistry at 1, 2, 4 and 8 days after AIA. By using fluorescence microscopy and image analysis techniques, the mean gray intensity (MGI) was obtained to evaluate CGRP-, SP- and GLS-ir in each traced neuronal profile (NP). The cross-sectional area of the NPs also was measured and neurons were further classified into small-, medium- and large-sized groups. It was found that 1) there was an increase in the proportion and MGI of CGRP- and SP-containing neurons after inflammation; 2) the peak elevation of GLS-MGI was at 1 day after initiating AIA, and a significant elevation of GLS-ir was observed in small-sized CGRP- and SP-containing

DRG neurons; 3) as AIA progressed, some medium and large neurons became CGRP- and SP-positive. These results help further characterize the temporal alteration of glutamate metabolism regarding subpopulations of DRG neurons as they respond to peripheral inflammation.

Introduction

Dorsal root ganglia (DRG) neurons are primary sensory neurons that detect and convey somatosensory information from peripheral tissue to the central nervous system (CNS). Most if not all DRG neurons use glutamate as the neurotransmitter (Miller et al., 1993, Bulling et al., 2001, Miller et al., 2011), and glutamate receives accumulative attention for its role in nociception transmission and sensitization. During peripheral tissue injury or inflammation, elevated glutamate is found at peripheral terminals in the target tissues, such as knee joint, skin and muscle (Omote et al., 1998, Lawand et al., 2000), where glutamate contributes to excitation and sensitization of nociceptors (Du et al., 2001). During peripheral inflammation, elevated glutamate release occurs at the central terminals in the spinal dorsal horn (al-Ghoul et al., 1993, Zahn et al., 2002), where the central release of glutamate activates excitatory glutamate receptors on post-synaptic neurons for nociceptive neurotransmission, leading to sensitization of central post-synaptic neurons (Zahn et al., 2002, Inquimbert et al., 2012). Prolonged exposure to repetitive or continuous noxious stimulation from the peripheral terminal of primary afferents leads to adaptation in the neurochemical components of the DRG neurons, a cellular process termed a “phenotypic switch” (Woolf and Ma, 2007).

The direct source of excessive glutamate released from primary afferents during peripheral inflammation, however, has not been fully identified. At the synapses of glutamatergic neurons, the homeostasis of glutamate is maintained by the “glutamate-glutamine cycle”. In the glutamate-glutamine cycle, glutaminase is the most crucial synthetic enzyme that converts glutamine to glutamate and is the major source replenishing the glutamate neurotransmitter pool in presynaptic

terminals (Waagepetersen et al., 2005, Miller et al., 2011). Several studies in CNS neurodegenerative diseases indicate that dysregulated glutaminase is related to excessive glutamate production and contributes to neuronal excitotoxicity found in cerebral ischemia, Alzheimer's disease and HIV-associated neurocognitive disease (HAND) (Newcomb et al., 1998, Burbaeva et al., 2005, Potter et al., 2013). In the peripheral nervous system, glutaminase expression and enzyme activity are elevated in DRG neuronal cell bodies and at peripheral terminals during chronic adjuvant-induced arthritis (Miller et al., 2012), and peripheral inhibition of glutaminase produces long-term suppression of edema and nociceptive behaviors in both adjuvant- and carrageenan- induced inflammation (Hoffman and Miller, 2010, Miller 2007). These studies support the idea that elevated glutaminase in the primary afferents may lead to excessive production of glutamate at central and peripheral terminals, and thus contribute to inflammatory pain. To date, numerous studies have evaluated glutamate release and the response of glutamate receptors during inflammation (Miller et al., 2011), however, limited studies have been performed to assess the response of glutamate metabolism, e.g., glutaminase levels, in primary afferents. Thus, the goal of the present studies is to determine the temporal response of glutaminase in subpopulations of cell bodies of DRG neurons. By using fluorescence microscopy and image analysis techniques, we categorize each neuronal profile (NP) by cross-sectional area and evaluate the alteration of glutaminase immunoreactivity in those populations of neurons containing calcitonin gene-related peptide (CGRP) and substance P (SP), two neurochemical markers for peptide-containing nociceptive neurons (Hanesch et al., 1993, Moussaoui et al., 1993, Snider and McMahon, 1998).

Materials and methods

Animals and adjuvant induced arthritis

Adult Sprague-Dawley rats (200 - 350g) of mixed sex (n=four/group, two male and two female) were housed on a 12:12 light/dark cycle, and given access to food and water *ad libitum*. Rats

were anesthetized with isoflurane (5% induction, 2% maintenance) before any injection. Adjuvant-induced arthritis (AIA) was induced by a single unilateral subcutaneous injection of 150 µl of 1:1 saline/CFA emulsion (complete Freund's adjuvant, 75 µg/150 µl) in the rat hindpaw plantar surface. AIA was allowed to developed for eight, four, two, and one days prior to tissue collection so that DRGs from all time points were collected on the same day. Naïve SD rats with no injection were used as control and processed on the same day with the experimental groups. Procedures were carried out in accordance with the National Institut of Health Guide for the care and use of laboratory animals and approved by the committee of animal care and use for research at Oklahoma State University Center for Health Sciences. All efforts were made to minimize the number of animals used and their suffering.

Immunohistochemistry

In the present study, all tissue collections were performed on the same day of experiment and processed for immunohistochemistry under the same conditions to minimize batch-to-batch variations introduced during tissue handling and processing. Rats were anesthetized with 3 ml of Avertin (2.5% of 2, 2, 2-Tribromoethanol, Sigma-Aldrich) and 0.8 ml of xylazine (1 mg/ml, AnaSed, LLOYD). Transcardial perfusion was performed by the following procedures. The abdomen and thoracic cavities were opened surgically and a perfusion cannula was inserted through a cut in the left ventricle into the ascending aorta. The cannula was fixed in place by clamping a straight hemostat across the superior part of the ventricles and then the right atrium was cut open. Calcium free Tyrode's solution, 80 ml, was pumped through the cannula followed by 300 ml optimized fixative for GLS immunolabeling described in previous papers (Hoffman et al., 2010). Briefly, it contains 0.2% (w/v) paraformaldehyde, 70% (v/v) picric acid, 0.1M sodium phosphate buffer at pH 7.3 at room temperature. Ipsilateral L4 DRGs were removed and post-fixed in the same fixative for 4 h at 4°C. Tissues were transferred to 10% sucrose in PBS, pH 7.3 overnight at 4°C. Frozen sections were cut at 10 µm in a cryostat (HM550, Thermo Scientific,

USA). Every fifth section was used to reduce the possibility of double-counting the same neuron. Dried sections were rinsed three times with PBS and blocked in 0.5% (w/v) polyvinlypyrolideone and 0.5% bovine serum albumin in PBS with 0.3% (v/v) Triton X-100 (PBS-T, Sigma). Colocalization of glutaminase with CGRP or SP was performed using fluorescence immunohistochemistry. The polyclonal rabbit anti-glutaminase antiserum was a generous gift from Dr. Norman Curthoys (Colorado State University). Sections were incubated in the antisera containing rabbit-anti glutaminase (1:20,000) with mouse anti-CGRP (1:8,000; Santa Cruz) or mouse anti-SP (1:2,000; R&D Systems) for four days at 4°C as described by Hoffman et al., 2010. After incubation in primary antisera, sections were rinsed three times in PBS and incubated in secondary antisera containing biotinylated goat anti-rabbit IgG (1.0 μg/ml; Vector Laboratories; Burlingame, CA, USA) and Alexa Flour 555 conjugated goat anti-mouse IgG (1.67 μg/ml; Invitrogen; Carlsbad, CA, USA) diluted in PBS-T for 1 h at room temperature. Sections were rinsed twice in PBS and once in sodium carbonate buffered saline (SCBS, pH 9.6) before incubating for 1 h in 1.0 μg/ml avidin-fluorescein isothiocyanate (FITC; Vector Laboratories) diluted in SCBS. After three rinses, sections were incubated in 300 nM 4',6-diamidino-2-phenylindole (DAPI) diluted in PBS for 10 min. After three PBS rinses, coverslips were apposed to slides with ProLong Gold Mounting Media (Invitrogen).

Image Analysis

Images were captured with a 40X objective on a BX51 epifluorescence microscope (Olympus; Center Valley, PA, USA) using a SPOT RT740 camera (Diagnostic Instruments; Sterling Heights, MI, USA). The micrographs were stored as 8-bit grayscale tiff format with the pixel intensity value ranging from 0-255. The exposure time and gain combination was determined empirically for each antigen in which the dimmest regions of tissue could be discerned visually for tracing, but the pixel values in brightest regions were not oversaturated. This approach allowed images to be evaluated along the linear aspect of immunofluorescence intensity. The exposure time and gain

combination for a single antigen was kept the same for all tissue sections from all animals. Five non-overlapping random fields of view were captured from at least three sections of each DRG separated by 50 μm. For each field of view, three filters were used for detection of each fluorophore: FITC (green), TRITC (Alexa Fluor 555, red), and DAPI (blue). The quantitative method to measure the immunoreactivity of each antigen has been described previously (Fang et al., 2005, Hoffman et al., 2010, Hoffman et al., 2011). Neuronal profiles (NP) were counted with the following criteria: 1) the NP was through the center of the cell with a visible nucleus that was detected in the DAPI filter; and 2) the NP had an intact cytoplasm that did not touch the edge of the image. NPs were traced with a Cintiq 21UX interactive pen display (Wacom; Kita Saitama-Gun, Saitama, Japan), using the freehand selection tool in ImageJ (National Institutes of Health; Bethesda, MD, USA). The cross-sectional area (μm^2) and mean gray intensity (MGI) of pixels in the cytoplasm (excluding the nucleus) was then recorded and measured as the region of interest (ROI). For CGRP- and SP-ir, the raw value of MGI was obtained with ImageJ software and analyzed, while a relative MGI was applied to assess the change of GLS-ir in all following analyses.

Our laboratory previously reported that all DRG neurons were immunoreactive for GLS immunolabeling using optimized fixation (Miller et al., 1993, Miller et al., 2002, Hoffman et al., 2011), so determining a GLS-MGI threshold was unnecessary for the current study. In order to combine GLS-MGI from both sets of tissue sections, double-labeled for GLS/CGRP and GLS/SP, the GLS-MGI (absolute MGI, designated as C) acquired for each NP was normalized on a 1 to 100 scale and combined to evaluate the immunoreactivity of GLS. To accomplish this, the GLS mean gray value of the most weakly labeled neuron in the CGRP- or SP-colabeled set (A) was determined as was the GLS mean gray value of the most intensely labeled neuron in each data set (B); each neuron was given a relative mean gray value=(100 × (C-A)/(B-A)). The relative MGI of GLS (GLS-rMGI) was used to quantitate the immunoreativity and estimate GLS expression (Hoffman, 2009, Hoffman et al., 2010).

Threshold MGI of CGRP- and SP-containing DRG neurons

A threshold SP and CGRP MGI was established to determine if a NP was to be considered immuno-positive (+ve) or –negative (-ve) for CGRP and SP, respectively (Fig. 1). The threshold MGI value of each antigen was determined from the naïve control group, since several studies reported that the proportion of neuropeptide-containing neurons could increase after peripheral inflammation (Hanesch and Schaible, 1995, Neumann et al., 1996, Woolf, 1996).

To determine the CGRP-MGI threshold, the CGRP-MGI of each NP was ranked from the smallest to the largest (Figure 1A). The rank and MGI were plotted in x- and y-axis respectively. A gap and change in shape and slope of the curve were observed around an MGI of 10 from the graph (Fig. 1A). Neuronal ranking of MGI<10 produced a line with gradual slope and these neurons mainly represented the background or non-specific immunolabeling in the DRG tissue sections, whereas neuronal ranking with MGI≥10, was represented by a curve indicating a rapid increase in MGI of each NP. This threshold (MGI≥10) was re-evaluated in a scatter plot with CGRP-MGI plotted against the cross-sectional area (Fig. 1B). The scatter plot showed that CGRP+ve NPs have cross-sectional area in small- (<400 μm^2; diameter, 23 μm) and medium- (400-800 μm^2; diameter, 23-32 μm) sized ranges, with a few falling in large-sized (>800 μm^2; diameter, 32μm) range (Fang et al., 2005, Hoffman et al., 2010). The proportion of CGRP+ve NPs among total NPs was 39.8%, which is in agreement with previous studies (Lawson, 1995, Lawson et al., 2002). A similar method was applied to determine the threshold MGI of SP (Figure 1C, 1D), and SP MGI threshold was set at 20. The proportion of SP+ve DRG neurons in naïve animals was 27.5 %, which is in accordance with the reported ratio 18-25% (Lawson, 1995, Lawson et al., 1997). The scatterplot of SP has a clearer distribution pattern than that of CGRP (Fig. 1B, 1D), demonstrating an inverse relationship between cell area and MGI of each NP. Most of the intensely labeled SP NPs have cross-sectional area between 0-400 μm^2 with smaller numbers of NPs in the medium-sized category. NPs with an area above 800 μm^2 were not labeled. This distribution pattern is in accordance with the notion that SP is mainly expressed in small

nociceptive neurons. The threshold MGI value of CGRP (MGI≥10) and SP (≥20) then was applied to determine if a NP was to be considered as CGRP+ve or SP+ve in the inflamed groups.

Statistics

Data of MGI from the image analyses were reported in following orders in the Results section unless other indicated: as mean ± standard error of the mean (SEM), median and percentage change in median compared to naïve control animals. A total of 3489 neuronal profiles (NPs; 2107 co-labeled with CGRP and 1382 co-labeled with SP) were traced and analyzed in the present study. All data were subjected to the Kolmogorov-Smirnov test to determine the normality of the distribution and homoscedasticity of the population. MGI values were not normally distributed and variances were heterogenous for nearly all comparisons. Nonparametric statistical analyses, therefore, were applied and the median is shown in the vertical scatter plots. Significant differences in MGI among different experimental conditions were determined by the non-parametric one-way ANOVA (Kruskal-Wallis test) by ranks followed by *Dunn post hoc* test (Prism version 5.03, GraphPad Software Inc., La Jolla, CA). In all analyses, *p*-value less than 0.05 were considered significant. * $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$.

Results

Double-label immunohistochemistry was performed to visualize the colocalization of GLS with CGRP or SP in adult rat DRG sections from naïve control and inflamed animals. Representative images from ipsilateral L4 DRG after 1, 2, 4, and 8 days of inflammation are shown compared to naïve animals (Fig. 2). In naïve animals, CGRP-ir was observed in small, medium and some large neurons (Fig. 2A'-2E'), and SP-ir (Fig. 2F'-2J') was almost exclusively found in small to medium-sized neurons. By setting an MGI threshold for CGRP- and SP-ir, it was found that in the naïve control group, 39.8% of the NPs were considered CGRP positive (Fig. 1A, 1B, 3A) and SP labeled 27.5% of the NPs (Fig. 1C, 1D, 3B). These proportions are in the range mentioned in

previous reports (Lawson, 1995, Lawson et al., 1997, Lawson et al., 2002). GLS-ir was detected in all DRG NPs (Fig. 2A-2J), and thus all CGRP- and SP-containing neurons were also immunoreative for GLS (Fig. 2A"-2J"). No detectable GLS-ir was observed in satellite cells or Schwann cells in DRG sections after AIA, which confirmed previous studies that glutaminase was confined to the DRG neuronal compartment (Miller et al., 1993, Miller et al., 2002).

Temporal alteration of proportion and MGI of CGRP and SP neurons during adjuvant-induced arthritis (AIA)

To determine if AIA resulted in altered immunoreactivity in CGRP-labeled neurons, the proportion of CGRP+ve NPs and CGRP-MGI in CGRP+ve NPs were evaluated. At 1 day AIA, the proportion of small-sized CGRP+ve neurons increase (~10%) while that of medium-sized CGRP+ve neurons decrease (~5%; Fig. 3A). There was a 16% increase in the overall proportion of CGRP-containing NPs at 4 days AIA, and no significant alteration was observed in the overall proportion of CGRP+ neurons at other time points during AIA. When further evaluating the NPs by cross-sectional area groups at 4 AIA, small-, medium- and large-sized NPs all contributed to the increased proportion of CGRP-labeled neurons at this time point (Fig. 3A).

AIA produced a robust and persistent elevation of the proportion of SP-containing NPs during the acute phase, which reached maximum at 2 days after CFA-injection and remained elevated through 8 days of AIA (Fig. 3B). When further dividing the NPs by cross-sectional area, it was found that during the acute phase of AIA (1-2 d), the small neurons are the major contributors to the increased proportion of SP+ve NPs (~10%). During the chronic phase of AIA (4-8 d), the proportion of medium SP+ve NPs increased (~10%) compared to naïve control group, and more large neurons had above-threshold SP-ir following AIA (Fig. 3B).

When evaluating CGRP- and SP-MGI, CGRP- (Fig. 4A) and SP- (Fig. 4E) MGI at 4 days AIA were significantly elevated compared to that at 1 and 8 days AIA. No statistical difference was found at any day following AIA compared to the naïve animals in CGRP- or SP-labeled NPs.

When the NPs were further divided into small, medium and large sizes, the elevated CGRP- and SP-MGI occurred in small CGRP- (Fig. 4B) and SP- (Fig. 4F) containing NPs at 4 days AIA.

Temporal alteration of relative GLS-MGI in all DRG NPsand CGRP-, and SP-containing NPs during AIA

NPs double-labeled for GLS/CGRP and GLS/SP were combined to quantitatively assess the temporal alteration of GLS immunoreactivity (-ir) in DRG neuronal profiles (NPs). A total of 3489 neuronal profiles (NPs; 2107 co-labeled with CGRP and 1382 co-labeled with SP) were traced and analyzed (Fig. 5A-5D).

An increase of relative GLS-MGI (GLS-rMGI) occurred at 1 day AIA (mean = 49.4 ± 0.6; median = 47.4; 18.6%; P<0.001) compared to naïve animals (mean = 40.5 ± 0.5; median = 40.0), and remained elevated at 2 days AIA (mean = 45.9 ± 0.5; median = 45.0; 12.7%; P<0.001). At 4 days AIA (mean = 35.2 ± 0.5; median = 34.2; 14.5%; P< 0.001), GLS-ir was significantly below basal level but returned to basal level at 8 days (Fig. 5A). To quantify the alteration of relative GLS-MGI regarding DRG cell sizes, GLS-MGI NPs were separated by cross-sectional area. It was found that the GLS-rMGI of small- (<400 μm^2, Fig. 5B) and medium-sized (400-800 μm^2, Fig. 5C) NPs followed similar temporal alteration pattern and magnitude found in total NPs. In large (>800 μm^2; Fig. 5D) NPs, a significant increase occurred only at 1 day AIA (mean = 44.1 ± 1.2; median = 44.5; 20.6%, P< 0.001) compared to large NPs from control group (mean = 36.8 ± 1.2; median = 36.9).

To further explore temporal alteration of GLS in nociceptive neurons, we evaluated GLS-rMGI in CGRP- and SP-labeled DRG NPs (Fig. 5E-5H, 5I-5L). GLS-rMGI in total traced CGRP-containing NPs (Fig. 5E) showed the similar temporal alteration pattern found in all traced NPs (Fig. 5A). An elevation occurred at 1 day (mean = 52.7 ± 1.0; median = 50.7; 20.4%, P< 0.001) after AIA compared to control (mean = 41.9 ± 1.0; median = 42.1). A 4 &8 days, GLS-rMGI was below basal level. When the neuronal profiles were further divided by size, GLS-ir in small,

medium and large CGRP+ve neurons followed the general alteration pattern that was observed in GLS-ir in total traced CGRP+ve neurons. The peak GLS-ir was observed at 1 day AIA in small- (mean = 51.5 ± 1.2; median = 49.6; 39.3%, $P< 0.001$; Fig. 5F) and medium-sized (mean = 56.4 ± 2.3; median = 55.1; 19.0%, $P< 0.001$) CGRP+ve neurons, followed by a decrease during the chronic phase of AIA (Fig. 5G). Significant decrease in the GLS-ir in large CGRP+ve neurons was observed at 4 and 8 days compared to 1 day following inflammation (~33%). The overall response pattern followed that observed in small- and medium-sized neurons (Fig. 5H).

The peak elevation of GLS-ir in all traced SP+ve neurons showed a biphasic pattern, different from CGRP+ve NPs. An acute increase of GLS-ir was observed 1 day AIA (mean = 48.5 ± 2.1; median = 46.4; 28.7%, $P< 0.001$) and reached a first peak at 2 days AIA (mean = 49.8 ± 1.1; median = 49.8; 38.3%, $P< 0.001$), followed by a significant drop to basal level at four days AIA, while a second peak was observed at eight days AIA (mean = 50.1 ± 1.5; median = 48.5; 34.7%, $P< 0.001$) after AIA (Fig. 5I). When the neuronal profiles were further divided by size, GLS-ir in small SP+ve neurons followed a similar biphasic elevation observed in GLS-ir in total traced SP+ve neurons, with maximum elevations at 1, 2 and 8 days and a drop in MGI to basal immunoreactivity at 4 days AIA (Fig. 5J). In large SP+ve neurons, GLS-ir was significantly increased compared to those of 1 day (~30%) and 4 day (~26%) following AIA respectively (Fig. L).

Discussion

In the present study, by co-labeling GLS with neuropeptides and separating neurons by cross-sectional area, we further evaluated the temporal alteration of GLS in small, medium and large sized CGRP- and SP-containing DRG neurons as they respond to peripheral inflammation. It was found that: 1) The proportion, but rarely the MGI of CGRP- and SP-containing DRG neurons were up-regulated during peripheral inflammation and some large neurons express detectable CGRP- and SP-ir during AIA; 2) GLS was significantly elevated at 1 and 2 days after AIA; 3)

GLS was elevated in small-sized CGRP+ve and SP+ve DRG neurons during the acute phase (1-2 d) of AIA and in SP+ve DRG neurons during the chronic phase (8 d) of AIA. These data further support the notion that GLS levels increase in response to peripheral inflammation and increased GLS may be involved in the development and maintenance of inflammatory pain via enhancing glutamate metabolism in primary afferents (Miller et al., 2011, 2012).

The proportion and MGI of SP- and CGRP-containing DRG neurons are altered during AIA

CGRP and SP are neuropeptides that are synthesized in and released by subpopulations of DRG neurons and have received extensive study for their roles in experimental and clinical inflammation and pain. Upon peripheral release, CGRP and SP cause increased arteriole vasodilation and capillary venular permeability in inflamed tissue, and are generally accepted as the initiators of neurogenic inflammation (Moussaoui et al., 1993, Holzer, 1998). Numerous quantitative studies have reported the temporal response of CGRP and SP in DRG neuronal cell body at different phases of peripheral inflammation for messenger RNA and protein levels. During inflammation, a rapid transcription occurs of α-CGRP and β-preprotachykinin-A (β-PPT-A)genes, major genes encoding isoforms of CGRP and SP protein respectively, in DRG neurons as early as 30 min post CFA inoculation and these levels stay up-regulated over weeks (Noguchi et al., 1988, Donaldson et al., 1992, Bulling et al., 2001). CGRP and SP protein expression also is up-regulated in DRG cell bodies during both acute and chronic phases of inflammation (Donnerer et al., 1992, Hanesch et al., 1993, Galeazza et al., 1995). Though the time point of the maximum gene induction and protein synthesis varies from study to study due to different experimental designs and conditions, all these data indicate that peripheral inflammation causes the up-regulation of neuropeptide production in the DRG neuronal cell body. In the present study, it was found that an increase in proportion of CGRP-containing NPs occurred at 4 days after AIA, while SP had an early-onset increased proportion at 1 and 2 days after CFA injection, remaining elevated through 8 days. When further dividing the NPs by their cross-sectional area, it was found that during the acute phase of AIA (1 to 2 days), small-sized CGRP+ve and SP+ve neurons are

the major contributors to the increase in proportion, while during the chronic phase of AIA, medium- and large-sized neurons started to express detectable CGRP-/SP-ir and contribute to the increased proportion during the chronic phase. The increased proportion of CGRP and SP are in accordance with previous studies showing that a subpopulation of DRG neurons, especially in the medium- to large-sized neurons, undergo a phenotypic switch by expressing detectable CGRP and SP (Donaldson et al., 1992, Woolf and Doubell, 1994, Neumann et al., 1996, Bulling et al., 2001). Neither CGRP- nor SP-MGI showed statistical differences at any day during AIA compared to the naïve animals, but a trend of increased accumulation of CGRP- and SP-MGI was observed at 4 days AIA. These results confirmed the observation from previous studies that peripheral inflammation activates the synthesis of CGRP and SP in a subpopulation of neurons in the ipsilateral ganglia, which under normal conditions either do not synthesize these neuropeptides or only synthesize them at a level that is below the threshold or sensitivity of the detection methods.

Results from radioimmunoassay (RIA) methods in previous studies using DRG homogenates established the general notion that peripheral inflammation stimulates an increases in the total amount of neuropeptides in the DRG neuronal cell bodies. These techniques, however, are unable to detect changes in individual neurons to determine if the increased amount comes from change in number of neurons expreing neuropeptides or change in protein expression per neuron (Donnerer et al., 1992, Smith et al., 1992, Nicholas et al., 1999). Immunohistochemistry and image analysis are techniques that permits assessment of the relative concentration of protein expression in individual neuronal cell bodies rather than the average concentration in a tissue homogenate. Furthermore, peptide content of peripheral or central axons within the DRG is excluded in image analysis compared to the RIA methodologies. Synthesis of CGRP and SP appears to be confined to the neuronal cell bodies, which makes axonal transport the exclusive source for delivery of neuropeptides to the peripheral and central nerve terminals (Harmar et al., 1980, Harmar and Keen, 1982). Because protein concentration detected of the cell body with

image analysis is a net outcome of protein synthesis and axonal transport, the temporal changes of CGRP- and SP-MGI reflect both processes. Previous studies found that up-regulation of CGRP and SP mRNA occurs as early as 30 min after CFA inoculation (Donaldson et al., 1992, Hanesch et al., 1993, Bulling et al., 2001). This potentially leads to elevated protein concentration at an earlier time than the first time point we chose to evaluate in the current study (24 h). Presumably, peripheral inflammation may increase anterograde transport of newly synthesized neuropeptides. The mechanism that drives the neuropeptide axonal transport to the nerve terminal is not fully elucidated, nor there is any solid correlation between neuronal discharge and axonal transport (Hammerschlag and Bobinski, 1992). Increased axonal transport, however, must be considered to affect from the temporal events occurring in the neuronal cell body following inflammation. Our present data indicate that neurons not producing SP or CGRP peptides under normal conditions have significant contribution to the increased levels of CGRP and SP in the DRG throughout different phases of inflammation. Hence, MGI of newly induced CGRP- and SP-expressing neurons may reach the MGI threshold, and thus dilute the average/median of the MGI in the inflamed animals. The possibilities listed above provide explanations for the differences between our observation and previous studies. Collectively, changes in DRG neuropeptide level in response to inflammation involve increased proportion of neurons, increased synthesis per neuron, and/or altered axonal transport.

AIA induces temporal alteration of GLS-MGI in SP+ve and CGRP+ve DRG neurons

Previous studies report that neurons have limited ability for glutamate *de novo* synthesis, so the major source that replenishes the glutamate neurotransmitter pool is via converting glutamine to glutamate by GLS. *In vivo* studies show that GLS is a neuronal enzyme and is considered as the marker for glutamatergic neurons (Kvamme et al., 2000, Kvamme et al., 2001, Waagepetersen et al., 2005). Acute peripheral inflammation leads to activation of transducer proteins and increased generation of transducer potentials (Caterina et al., 1997, Woolf and Ma, 2007). The Calcium influx via the transducer ion channels may mediate the synaptic release of glutamate in response

to increased neuronal activity. The calcium influx at the nerve terminal may also contribute to the activation of local glutaminase consistent with studies in rat brain synaptosomes showing that calcium activates GLS enzymatic activity (Kvamme et al., 2001). Increased GLS activity will lead to further production of glutamate to replenish glutamate that is released by neuronal discharge. During chronic pain, prolonged exposure to inflammatory stimulation from the peripheral terminal leads to adaptation in protein transcription, translation, and trafficking in the neuronal cell bodies (Woolf and Ma, 2007). Similar to the neuropeptides, DRG neuronal cell bodies are the sole synthetic site of elevated GLS, and the anterograde transport rate of GLS needs to be taken into consideration when considering neuronal cell body alterations. Our lab has previously reported the elevation of glutaminase immunoreactivity and enzyme activity in DRG neuronal cell bodies and inflamed hind paw skin at 7 days of AIA (Miller et al., 2012), but the altered amount of GLS is likely to be present earlier in the neuronal cell bodies in order to be transported to the inflamed site prior to 7 days. To better understand the temporal alteration of glutaminase in response to peripheral inflammation, the alteration of GLS during acute and chronic phases of AIA was evaluated. GLS showed an early increase during the acute phase of AIA (1-3 d), followed by a significant drop relative to basal GLS-ir of naïve control animals at 4 days and a return to basal level during the chronic phase of AIA (4-8 d). When dividing the neuronal profiles by cross-sectional area, it was found that the alteration occurred across all three sizes of neurons, but most notably in especially in small- to medium-sized neurons. Similar to neuropeptides, following translation of GLS in the neuronal cell bodies, GLS is transported to the peripheral- and central- terminals for glutamate production. The robust increase of GLS-ir during acute AIA suggests a significant fast response to peripheral inflammation by increasing the GLS protein synthesis. However, it could also reflect decreased protein degradation or decreased anterograde transport, either of which would elevate GLS in the cell body. Because neither of these is consistent with the observed increases in peripheral glutamate observed in other studies

(Lawand et al., 1997, Omote et al., 1998, Lawand et al., 2000), the increase of GLS-ir during acute AIA is from up-regulated protein synthesis.

Glutamate is co-released with CGRP and SP from central terminals of DRG neurons in the spinal cord (Merighi et al., 1991), and neuropeptides augment glutamate release and enhance the excitability of post-synaptic glutamatergic neurons in the nociceptive pathway (Kangrga et al., 1990, Okano et al., 1998). It was found that CGRP- and SP-containing neurons had an up-regulated GLS immunoreactivity at 1 day of AIA, and that the small- to medium-sized neurons are the major contributors to the elevated GLS. Studies using CGRP- and SP- knockout mice demonstrate that the two neuropeptides contribute to the development and maintenance of the hyperalgesia after peripheral inflammation including the development of nociceptive behaviors. However, hypersensitivity is not completely removed in $CGRP^{-/-}$ and $SP^{-/-}$ mices, which indicates there are other substances, neuronal cell populations, or mechanisms contributing to the detection of inflammatory pain. Both CGRP and SP facilitate the effects of glutamate at central terminal in the dorsal horn, and glutamate functions as a neurotransmitter at the central terminal and a sensitizer at the peripheral terminal in response to inflammation. Given the elevation in the proportion of SP-containing neurons and the prolonged elevation of GLS in this specific subpopulation, it is demonstrated that a subset of DRG neurons are specifically affected by changes related to peripheral inflammation. Elevated CGRP- and SP-ir are observed at the nerve terminals during inflammation (Donnerer et al., 1992, Nahin and Byers, 1994). In these peptide-containing neurons, elevated GLS was also observed which could lead to excess production of glutamate in nerve terminals. CGRP and SP facilitate the release of glutamate from the dorsal horn neurons (Otsuka and Konishi, 1976, De Biasi and Rustioni, 1988, Kangrga et al., 1990, Kangrga and Randic, 1990). These results show that in the peptide-containing nerve terminals, elevated neuropeptide concentration might lead to excess release of glutamate. Excessive glutamate release at the periphery could sensitize the nerve ending and lead to peripheral sensitization (Woolf and Ma, 2007); while the central glutamatergic synapses could be

strengthened by the co-release of the two neuropeptides with glutamate, which is considered as significant aspect of central sensitization (Ji et al., 2003, Latremoliere and Woolf, 2009). Elevated glutamate synthesis via altered GLS levels in the nerve terminals, therefore, would result in excessive nociception transmission leading to increased pain perception during inflammation.

Interestingly, the temporal alteration patterns of GLS were not identical between CGRP- and SP-labeled NPs. In CGRP-labeled NPs, the peak of elevated GLS-rMGI occurred at 1 day after inflammation, and returned to basal level at 2 to 8 days after inflammation. In SP+ve DRG NPs, GLS-rMGI showed a biphasic elevation at the acute phase and 8 days after AIA. The different temporal alteration patterns of GLS in CGRP- and SP-containing neurons may come from the different subpopulations labeled by SP and CGRP. Though CGRP and SP are generally considered having parallel localization and functions in the DRG neurons, CGRP is located in a larger proportion in DRG neurons with a wider distribution and projections at both central and peripheral terminals than those neurons containing SP. Electrophysiology studies show that in guinea-pig lumbar DRGs, SP-ir is limited to a subpopulation of nociceptive neurons, including both C- and A-δ fibers (Lawson et al., 1997), and only half of CGRP-containing neurons are nociceptive (Carlton and Hargett, 2002, Lawson, 2002). These studies indicate that SP may be a more specific neurochemical marker for nociceptors, while CGRP-containing neurons respond to more types of sensory stimuli than nociception (Lawson, 1995, Lawson et al., 2002).

The potential mechanisms underlying the changes in the temporal expression of glutaminase

Analyzing the temporal alteration patterns of GLS and neuropeptides in the DRG is essential to determine the functional and pathogenic role of different neurochemical components of sensory neurons in response to peripheral inflammation. These results show that peripheral inflammation produces a significant up-regulation in GLS expression. SP labels subsets of lightly myelinated Aδ- and unmyelinated C-nociceptors, In SP-containing DRG neurons, GLS elevation was observed during both acute and chronic phases in in the AIA model. This phenomenon indicates

that multiple regulators contribute to the elevated GLS at early and chronic phases. During the rapid onset and prolonged process of inflammation, altered neural electrical activity and retrograde transport of signaling molecules are two candidate mechanisms that drive the alteration in protein expression in nociceptive neuronal cells. Neural electrical activity and retrograde transport signaling molecules generated from the inflammation site are sent to DRG neuronal cell bodies and initiate a cascade of events including post-translational modification, transcription, translation, and trafficking of proteins, substances, or organelles that contribute to increased nociceptive transmission.

Peripheral sensitization, characterized by a reduced threshold for firing and an increase in the excitability of nociceptors, results in increased electrical neuronal activity of the DRG neurons, triggering a downstream signaling cascade in the neuronal cell bodies. Previous studies showed that adjuvant increases electrical activity in nerves innervating an injected join within 15 min. Blockade of the neural conduction with local anesthesia successfully blocks the rapid transcription of CGRP, PPT-A and μ-opioid receptors (MOR) mRNA in response to acute peripheral inflammation (Donaldson et al., 1994, Bulling et al., 2001, Puehler et al., 2004).These studies support the notion that increased neural discharge from the inflamed site contributes to the rapid initiation of transcription and protein synthesis in the DRG neuronal cell body. Short-term transient application of local anesthetic, however, does not block the late increase (3 days after AIA) of MOR mRNA after inflammation (Puehler et al., 2004). Whereas peripheral nerve section maintains an initial elevation of CGRP and SP in DRG neuronal cell bodies within 3 days post operation, but CGRP- and SP-ir decreases at 7 and 14 day (Zhang et al., 1993, Weissner et al., 2006). Electrophysiology studies using the AIA model showed that fundamental changes in membrane properties of nociceptive neurons occur at 2-4 days after onset of inflammation, and that the activity-dependent changes at 1-2 day after inflammation is incomplete (Djouhri and Lawson, 1999, Djouhri et al., 2001). These studies indicate that additional signaling events are

required to drive and maintain the post-acute alteration of protein synthesis and membrane properties after peripheral inflammation especially in the CGRP- and SP-expressing nociceptive neurons. Nerve growth factor (NGF) could be a candidate mediating the delayed effect in response to inflammation since retrograde transport from the periphery is required for long-term changes to occur. NGF not only participates in the development and maturation of nociceptive sensory systems, but also acts as an important link between inflammation, pain sensitization and axonal transport of proteins following peripheral CFA and other inflammatory agents(Woolf et al., 1994, Mendell et al., 1999, Woolf and Ma, 2007). NGF also contributes to the phenotypic switch of the DRG neurons during inflammation. During inflammation, both up-regulation in CGRP- and SP involves recruiting normally non-peptide expression neurons. In response to peripheral inflammation, SP-expression have been found in medium- to large-sized neurons from an initial distribution almost exclusively within A-δ and C fibers, and this expanded expression has been prevented using anti-NGF strategies (Neumann et al., 1996). Together with increased proportion of medium- and large-sized CGRP+ve neurons, these newly recruited CGRP+ve and SP+ve neurons increase the probability of augmented neurogenic inflammation (Donnerer et al., 1992, Maggi, 1995, Juranek and Lembeck, 1996) and increased glutamatergic synaptic strength at peripheral and central terminal, respectively (Otsuka and Konishi, 1976, De Biasi and Rustioni, 1988, Kangrga et al., 1990, Kangrga and Randic, 1990, Galeazza et al., 1995, Woolf, 1996). These well-established studies on CGRP and SP have supported both the activity-dependent and neurotrophin mechanisms based on the timescale of the acute and chronic phases of AIA, respectively. Hence, the alteration of GLS may also be under the regulation of the electrical neural activity and the target-derived neurotrophin.

The regulatory mechanisms for GLS expression in the nervous system are not fully understood. A regulatory model of GLS has been proposed using by Curthoys *et al.* using acute and chronic renal metabolic acidosis. An increase in rat renal GLS mRNAs has been characterized in response

to acute onset of acidosis following a 6-8 h lag and reaching a plateau within 16-18 h. The onset of acidosis leads to a gradual and cell-specific increase in the activity of mitochondrial GLS, and the increase in GLS activity during chronic acidosis results from an increase in the levels of total and translatable glutaminase mRNAs that results from an increased stability of the GLS mRNA. DRG neurons share the same GLS isoform as renal cells (Haser et al., 1985, Kvamme et al., 2001). The robust up-regulation of GLS-rMGI at 1 day indicates a fast signal in the DRG cell body after the onset of inflammation, suggesting that neuronal conduction might play an important role for the fast communication between neuronal cell body and peripheral terminal. Peripheral inflammation induces rapid increased neuronal activity in DRG neuronal cell bodies, which may mimic the sudden decreased pH in the acute renal acidosis model. The increased activity in nociceptive neurons may induce post-translational activation of a pre-existing transcript factor or a GLS mRNA stabilizing protein that is responsible for increased the GLS mRNA transcription and stabilization, leading to increase GLS protein synthesis (Curthoys and Watford, 1995, Curthoys and Gstraunthaler, 2001). Additional experiments are required to test this model in DRG neurons.

Retrograde transport of NGF has profound effects on the delayed expression of neuropeptides, which may be required for the maintenance of chronic inflammation and hypersensitivity. NGF deprivation decreases glutaminase in the embryonic DRG via destroying the small to medium sized capsaicin-sensitive DRG neurons (McDougal et al., 1981, McDougal et al., 1983). Though NGF is not required for basal glutaminase expression in adult DRG neurons (Hoffman et al., 2011), additional studies are required to find out the role of NGF on GLS expression in the presence of peripheral inflammation. GLS is a mitochondrial enzyme and presumably transported to the nerve terminals in mitochondria. It has been reported that NGF increases the movement and positioning of mitochondria in the axon (Chada and Hollenbeck, 2003, 2004), which may be a signal that promotes the anterograde transport of newly synthesized GLS to the nerve terminal.

NGF also mediates the activation of p38, a mitogen-activated protein kinase (MAPK) activated by cellular stress and cytokines, which in turn leads to increased TRPV1 protein levels in nociceptors (Ji et al., 2002). The p38 pathway also is implicated as an important downstream signaling pathway that enhances the stabilization of GLS mRNA in the renal acidosis model (Curthoys and Gstraunthaler, 2001). In summary, NGF is a reasonable factor to study in regard to their potential regulatory effects on GLS expression during peripheral inflammation and more studies are needed to test this mechanism.

Conclusions

Increased nociceptive sensitivity during peripheral inflammation involves many molecular, cellular and signaling mechanisms in the primary sensory neurons in the DRG. In the present study, we confirmed previous studies that the proportion and immunoreactivity of CGRP- and SP-containing DRG neurons are up-regulated in the presence of peripheral inflammation, and that a phenotypic switch is observed in medium- and large- sized DRG neurons during AIA; 2) GLS had a significant elevation during the acute phase (1-2 d) after AIA; 3) elevated GLS was observed in small-sized CGRP+ve and SP+ve DRG neurons during acute phase of AIA and in SP+ve DRG neurons during the chronic phase of AIA.

Peripheral inflammation drives peripheral sensitization and modifications in DRG neuronal cell by producing retrograde signals in nociceptive neurons. These signals, e.g., action potentials or nerve growth factor/TrkA, activate or increase transcription of pro-nociceptive molecules, such as CGRP, SP and GLS, to augment both central and peripheral sensitization. Elevated GLS could increase glutamate production at the spinal cord synapse and augment synaptic transmission via co-release with SP and CGRP. These results help further characterize a subpopulation of DRG neurons as they respond to inflammatory conditions. They further support the notion that GLS increases in response to peripheral inflammation, and increased GLS and neuropeptides may be

involved in the development and maintenance of inflammatory pain via enhancing glutamate metabolism in primary afferents.

References

al-Ghoul WM, Volsi GL, Weinberg RJ, Rustioni A (1993) Glutamate immunocytochemistry in the dorsal horn after injury or stimulation of the sciatic nerve of rats. Brain research bulletin 30:453-459.

Bulling DG, Kelly D, Bond S, McQueen DS, Seckl JR (2001) Adjuvant-induced joint inflammation causes very rapid transcription of beta-preprotachykinin and alpha-CGRP genes in innervating sensory ganglia. Journal of neurochemistry 77:372-382.

Burbaeva G, Boksha IS, Tereshkina EB, Savushkina OK, Starodubtseva LI, Turishcheva MS (2005) Glutamate metabolizing enzymes in prefrontal cortex of Alzheimer's disease patients. Neurochemical research 30:1443-1451.

Carlton SM, Hargett GL (2002) Stereological analysis of Ca(2+)/calmodulin-dependent protein kinase II alpha -containing dorsal root ganglion neurons in the rat: colocalization with isolectin Griffonia simplicifolia, calcitonin gene-related peptide, or vanilloid receptor 1. The Journal of comparative neurology 448:102-110.

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

Chada SR, Hollenbeck PJ (2003) Mitochondrial movement and positioning in axons: the role of growth factor signaling. The Journal of experimental biology 206:1985-1992.

Chada SR, Hollenbeck PJ (2004) Nerve growth factor signaling regulates motility and docking of axonal mitochondria. Current biology : CB 14:1272-1276.

Curthoys NP, Gstraunthaler G (2001) Mechanism of increased renal gene expression during metabolic acidosis. American journal of physiology Renal physiology 281:F381-390.

Curthoys NP, Watford M (1995) Regulation of glutaminase activity and glutamine metabolism. Annual review of nutrition 15:133-159.

De Biasi S, Rustioni A (1988) Glutamate and substance P coexist in primary afferent terminals in the superficial laminae of spinal cord. Proc Natl Acad Sci U S A 85:7820-7824.

Djouhri L, Dawbarn D, Robertson A, Newton R, Lawson SN (2001) Time course and nerve growth factor dependence of inflammation-induced alterations in electrophysiological membrane properties in nociceptive primary afferent neurons. The Journal of neuroscience : the official journal of the Society for Neuroscience 21:8722-8733.

Djouhri L, Lawson SN (1999) Changes in somatic action potential shape in guinea-pig nociceptive primary afferent neurones during inflammation in vivo. The Journal of physiology 520 Pt 2:565-576.

Donaldson LF, Harmar AJ, McQueen DS, Seckl JR (1992) Increased expression of preprotachykinin, calcitonin gene-related peptide, but not vasoactive intestinal peptide messenger RNA in dorsal root ganglia during the development of adjuvant monoarthritis in the rat. Brain research Molecular brain research 16:143-149.

Donaldson LF, McQueen DS, Seckl JR (1994) Local anaesthesia prevents acute inflammatory changes in neuropeptide messenger RNA expression in rat dorsal root ganglia neurons. Neuroscience letters 175:111-113.

Donnerer J, Schuligoi R, Stein C (1992) Increased content and transport of substance P and calcitonin gene-related peptide in sensory nerves innervating inflamed tissue: evidence for a regulatory function of nerve growth factor in vivo. Neuroscience 49:693-698.
Du J, Koltzenburg M, Carlton SM (2001) Glutamate-induced excitation and sensitization of nociceptors in rat glabrous skin. Pain 89:187-198.
Fang X, Djouhri L, McMullan S, Berry C, Okuse K, Waxman SG, Lawson SN (2005) trkA is expressed in nociceptive neurons and influences electrophysiological properties via Nav1.8 expression in rapidly conducting nociceptors. The Journal of neuroscience : the official journal of the Society for Neuroscience 25:4868-4878.
Galeazza MT, Garry MG, Yost HJ, Strait KA, Hargreaves KM, Seybold VS (1995) Plasticity in the synthesis and storage of substance P and calcitonin gene-related peptide in primary afferent neurons during peripheral inflammation. Neuroscience 66:443-458.
Hammerschlag R, Bobinski J (1992) Does nerve impulse activity modulate fast axonal transport? Molecular neurobiology 6:191-201.
Hanesch U, Pfrommer U, Grubb BD, Schaible HG (1993) Acute and chronic phases of unilateral inflammation in rat's ankle are associated with an increase in the proportion of calcitonin gene-related peptide-immunoreactive dorsal root ganglion cells. The European journal of neuroscience 5:154-161.
Hanesch U, Schaible HG (1995) Effects of ankle joint inflammation on the proportion of calcitonin gene-related peptide (CGRP)-immunopositive perikarya in dorsal root ganglia. Progress in brain research 104:339-347.
Harmar A, Keen P (1982) Synthesis, and central and peripheral axonal transport of substance P in a dorsal root ganglion-nerve preparation in vitro. Brain research 231:379-385.
Harmar A, Schofield JG, Keen P (1980) Cycloheximide-sensitive synthesis of substance P by isolated dorsal root ganglia. Nature 284:267-269.
Haser WG, Shapiro RA, Curthoys NP (1985) Comparison of the phosphate-dependent glutaminase obtained from rat brain and kidney. The Biochemical journal 229:399-408.
Hoffman EM (2009) The role of dorsal root ganglion glutaminase in nociception - A novel therapeutic target for inflammatory pain. In: Ann Arbor: ProQuest/UMI.
Hoffman EM, Miller KE (2010) Peripheral inhibition of glutaminase reduces carrageenan-induced Fos expression in the superficial dorsal horn of the rat. Neuroscience letters 472:157-160.
Hoffman EM, Schechter R, Miller KE (2010) Fixative composition alters distributions of immunoreactivity for glutaminase and two markers of nociceptive neurons, Nav1.8 and TRPV1, in the rat dorsal root ganglion. J Histochem Cytochem 58:329-344.
Hoffman EM, Zhang Z, Anderson MB, Schechter R, Miller KE (2011) Potential mechanisms for hypoalgesia induced by anti-nerve growth factor immunoglobulin are identified using autoimmune nerve growth factor deprivation. Neuroscience 193:452-465.
Holzer P (1998) Neurogenic vasodilatation and plasma leakage in the skin. General pharmacology 30:5-11.
Inquimbert P, Bartels K, Babaniyi OB, Barrett LB, Tegeder I, Scholz J (2012) Peripheral nerve injury produces a sustained shift in the balance between glutamate release and uptake in the dorsal horn of the spinal cord. Pain 153:2422-2431.
Ji RR, Kohno T, Moore KA, Woolf CJ (2003) Central sensitization and LTP: do pain and memory share similar mechanisms? Trends Neurosci 26:696-705.
Ji RR, Samad TA, Jin SX, Schmoll R, Woolf CJ (2002) p38 MAPK activation by NGF in primary sensory neurons after inflammation increases TRPV1 levels and maintains heat hyperalgesia. Neuron 36:57-68.
Juranek I, Lembeck F (1996) Evidence for the participation of glutamate in reflexes involving afferent, substance P-containing nerve fibres in the rat. Br J Pharmacol 117:71-78.

Kangrga I, Larew JS, Randic M (1990) The effects of substance P and calcitonin gene-related peptide on the efflux of endogenous glutamate and aspartate from the rat spinal dorsal horn in vitro. Neuroscience letters 108:155-160.
Kangrga I, Randic M (1990) Tachykinins and calcitonin gene-related peptide enhance release of endogenous glutamate and aspartate from the rat spinal dorsal horn slice. The Journal of neuroscience : the official journal of the Society for Neuroscience 10:2026-2038.
Kvamme E, Roberg B, Torgner IA (2000) Phosphate-activated glutaminase and mitochondrial glutamine transport in the brain. Neurochemical research 25:1407-1419.
Kvamme E, Torgner IA, Roberg B (2001) Kinetics and localization of brain phosphate activated glutaminase. Journal of neuroscience research 66:951-958.
Latremoliere A, Woolf CJ (2009) Central sensitization: a generator of pain hypersensitivity by central neural plasticity. J Pain 10:895-926.
Lawand NB, McNearney T, Westlund KN (2000) Amino acid release into the knee joint: key role in nociception and inflammation. Pain 86:69-74.
Lawson SN (1995) Neuropeptides in morphologically and functionally identified primary afferent neurons in dorsal root ganglia: substance P, CGRP and somatostatin. Progress in brain research 104:161-173.
Lawson SN (2002) Phenotype and function of somatic primary afferent nociceptive neurones with C-, Adelta- or Aalpha/beta-fibres. Exp Physiol 87:239-244.
Lawson SN, Crepps B, Perl ER (2002) Calcitonin gene-related peptide immunoreactivity and afferent receptive properties of dorsal root ganglion neurones in guinea-pigs. The Journal of physiology 540:989-1002.
Lawson SN, Crepps BA, Perl ER (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.
Maggi CA (1995) Tachykinins and calcitonin gene-related peptide (CGRP) as co-transmitters released from peripheral endings of sensory nerves. Progress in neurobiology 45:1-98.
McDougal DB, Jr., Yu MJ, Gorin PD, Johnson EM, Jr. (1981) Transported enzymes in sciatic nerve and sensory ganglia of rats exposed to maternal antibodies against nerve growth factor. Journal of neurochemistry 36:1847-1852.
McDougal DB, Jr., Yuan MJ, Dargar RV, Johnson EM, Jr. (1983) Neonatal capsaicin and guanethidine and axonally transported organelle-specific enzymes in sciatic nerve and in sympathetic and dorsal root ganglia. The Journal of neuroscience : the official journal of the Society for Neuroscience 3:124-132.
Mendell LM, Albers KM, Davis BM (1999) Neurotrophins, nociceptors, and pain. Microscopy research and technique 45:252-261.
Merighi A, Polak JM, Theodosis DT (1991) Ultrastructural visualization of glutamate and aspartate immunoreactivities in the rat dorsal horn, with special reference to the co-localization of glutamate, substance P and calcitonin-gene related peptide. Neuroscience 40:67-80.
Miller KE, Balbas JC, Benton RL, Lam TS, Edwards KM, Kriebel RM, Schechter R (2012) Glutaminase immunoreactivity and enzyme activity is increased in the rat dorsal root ganglion following peripheral inflammation. Pain Res Treat 2012:414697.
Miller KE, Douglas VD, Kaneko T (1993) Glutaminase immunoreactive neurons in the rat dorsal root ganglion contain calcitonin gene-related peptide (CGRP). Neuroscience letters 160:113-116.
Miller KE, Hoffman EM, Sutharshan M, Schechter R (2011) Glutamate pharmacology and metabolism in peripheral primary afferents: physiological and pathophysiological mechanisms. Pharmacol Ther 130:283-309.

Miller KE, Richards BA, Kriebel RM (2002) Glutamine-, glutamine synthetase-, glutamate dehydrogenase- and pyruvate carboxylase-immunoreactivities in the rat dorsal root ganglion and peripheral nerve. Brain research 945:202-211.
Moussaoui S, Carruette A, Garret C (1993) Further evidence that substance P is a mediator of both neurogenic inflammation and pain: two phenomena inhibited either by postsynaptic blockade of NK1 receptors or by presynaptic action of opioid receptor agonists. Regul Pept 46:424-425.
Nahin RL, Byers MR (1994) Adjuvant-induced inflammation of rat paw is associated with altered calcitonin gene-related peptide immunoreactivity within cell bodies and peripheral endings of primary afferent neurons. The Journal of comparative neurology 349:475-485.
Neumann S, Doubell TP, Leslie T, Woolf CJ (1996) Inflammatory pain hypersensitivity mediated by phenotypic switch in myelinated primary sensory neurons. Nature 384:360-364.
Newcomb R, Pierce AR, Kano T, Meng W, Bosque-Hamilton P, Taylor L, Curthoys N, Lo EH (1998) Characterization of mitochondrial glutaminase and amino acids at prolonged times after experimental focal cerebral ischemia. Brain research 813:103-111.
Nicholas RS, Winter J, Wren P, Bergmann R, Woolf CJ (1999) Peripheral inflammation increases the capsaicin sensitivity of dorsal root ganglion neurons in a nerve growth factor-dependent manner. Neuroscience 91:1425-1433.
Noguchi K, Morita Y, Kiyama H, Ono K, Tohyama M (1988) A noxious stimulus induces the preprotachykinin-A gene expression in the rat dorsal root ganglion: a quantitative study using in situ hybridization histochemistry. Brain research 464:31-35.
Okano K, Kuraishi Y, Satoh M (1998) Involvement of spinal substance P and excitatory amino acids in inflammatory hyperalgesia in rats. Japanese journal of pharmacology 76:15-22.
Omote K, Kawamata T, Kawamata M, Namiki A (1998) Formalin-induced release of excitatory amino acids in the skin of the rat hindpaw. Brain research 787:161-164.
Otsuka M, Konishi S (1976) Substance P and excitatory transmitter of primary sensory neurons. Cold Spring Harbor symposia on quantitative biology 40:135-143.
Potter MC, Figuera-Losada M, Rojas C, Slusher BS (2013) Targeting the Glutamatergic System for the Treatment of HIV-Associated Neurocognitive Disorders. Journal of neuroimmune pharmacology : the official journal of the Society on NeuroImmune Pharmacology.
Puehler W, Zollner C, Brack A, Shaqura MA, Krause H, Schafer M, Stein C (2004) Rapid upregulation of mu opioid receptor mRNA in dorsal root ganglia in response to peripheral inflammation depends on neuronal conduction. Neuroscience 129:473-479.
Smith GD, Harmar AJ, McQueen DS, Seckl JR (1992) Increase in substance P and CGRP, but not somatostatin content of innervating dorsal root ganglia in adjuvant monoarthritis in the rat. Neuroscience letters 137:257-260.
Snider WD, McMahon SB (1998) Tackling pain at the source: new ideas about nociceptors. Neuron 20:629-632.
Waagepetersen HS, Qu H, Sonnewald U, Shimamoto K, Schousboe A (2005) Role of glutamine and neuronal glutamate uptake in glutamate homeostasis and synthesis during vesicular release in cultured glutamatergic neurons. Neurochemistry international 47:92-102.
Weissner W, Winterson BJ, Stuart-Tilley A, Devor M, Bove GM (2006) Time course of substance P expression in dorsal root ganglia following complete spinal nerve transection. The Journal of comparative neurology 497:78-87.
Woolf CJ (1996) Phenotypic modification of primary sensory neurons: the role of nerve growth factor in the production of persistent pain. Philosophical transactions of the Royal Society of London Series B, Biological sciences 351:441-448.
Woolf CJ, Doubell TP (1994) The pathophysiology of chronic pain--increased sensitivity to low threshold A beta-fibre inputs. Current opinion in neurobiology 4:525-534.
Woolf CJ, Ma Q (2007) Nociceptors--noxious stimulus detectors. Neuron 55:353-364.

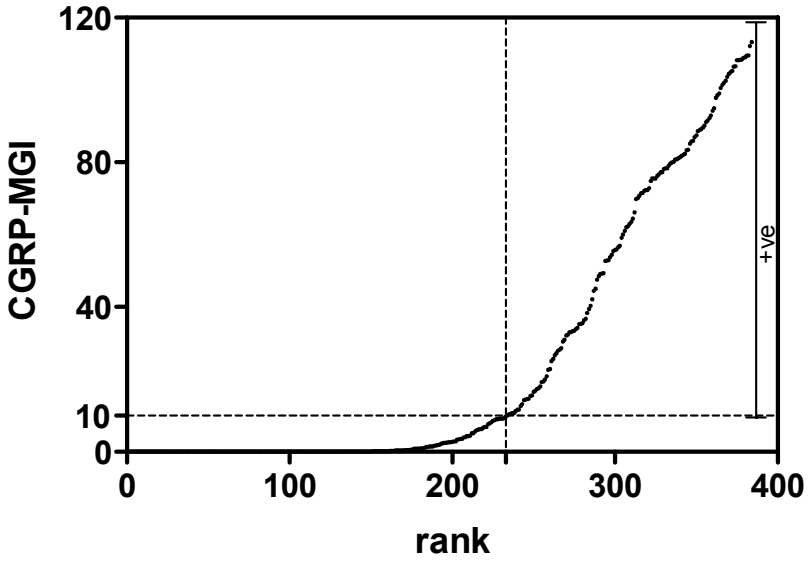
Woolf CJ, Safieh-Garabedian B, Ma QP, Crilly P, Winter J (1994) Nerve growth factor contributes to the generation of inflammatory sensory hypersensitivity. Neuroscience 62:327-331.

Zahn PK, Sluka KA, Brennan TJ (2002) Excitatory amino acid release in the spinal cord caused by plantar incision in the rat. Pain 100:65-76.

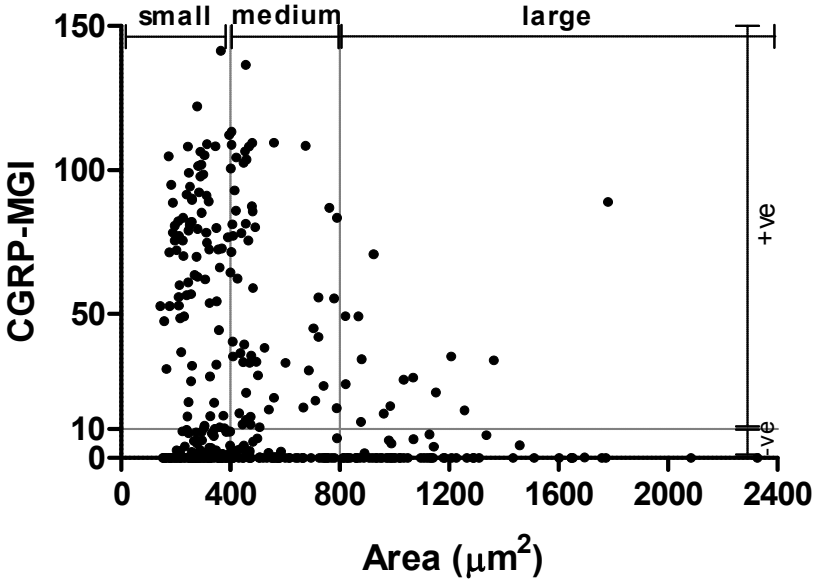
Zhang X, Ju G, Elde R, Hokfelt T (1993) Effect of peripheral nerve cut on neuropeptides in dorsal root ganglia and the spinal cord of monkey with special reference to galanin. Journal of neurocytology 22:342-381.

Figure 1

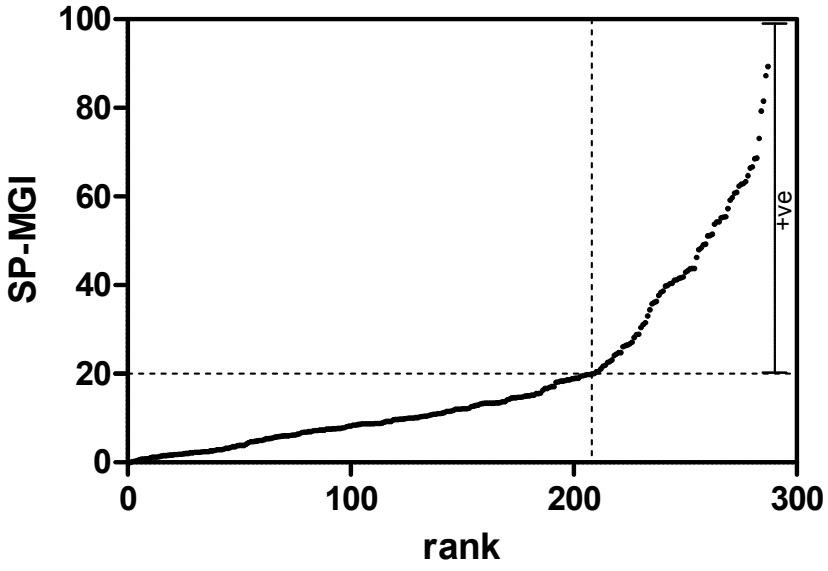
A.


120
80
40
10
0
CGRP-MGI
+ve
0
100
200
300
400
rank

B.


small
medium
large
150
100
50
10
0
CGRP-MGI
+ve
-ve
0
400
800
1200
1600
2000
2400
Area (μm²)

C.


100
80
60
40
20
0
SP-MGI
+ve
0
100
200
300
rank

D.

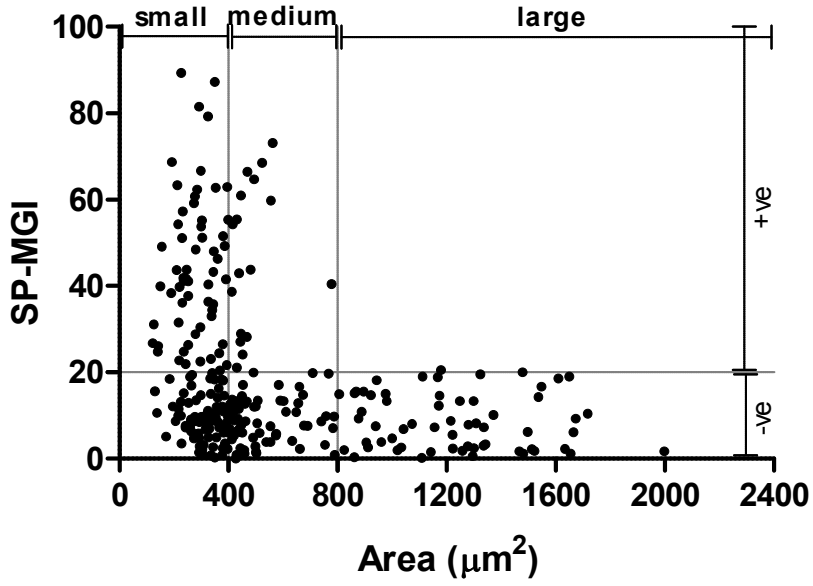
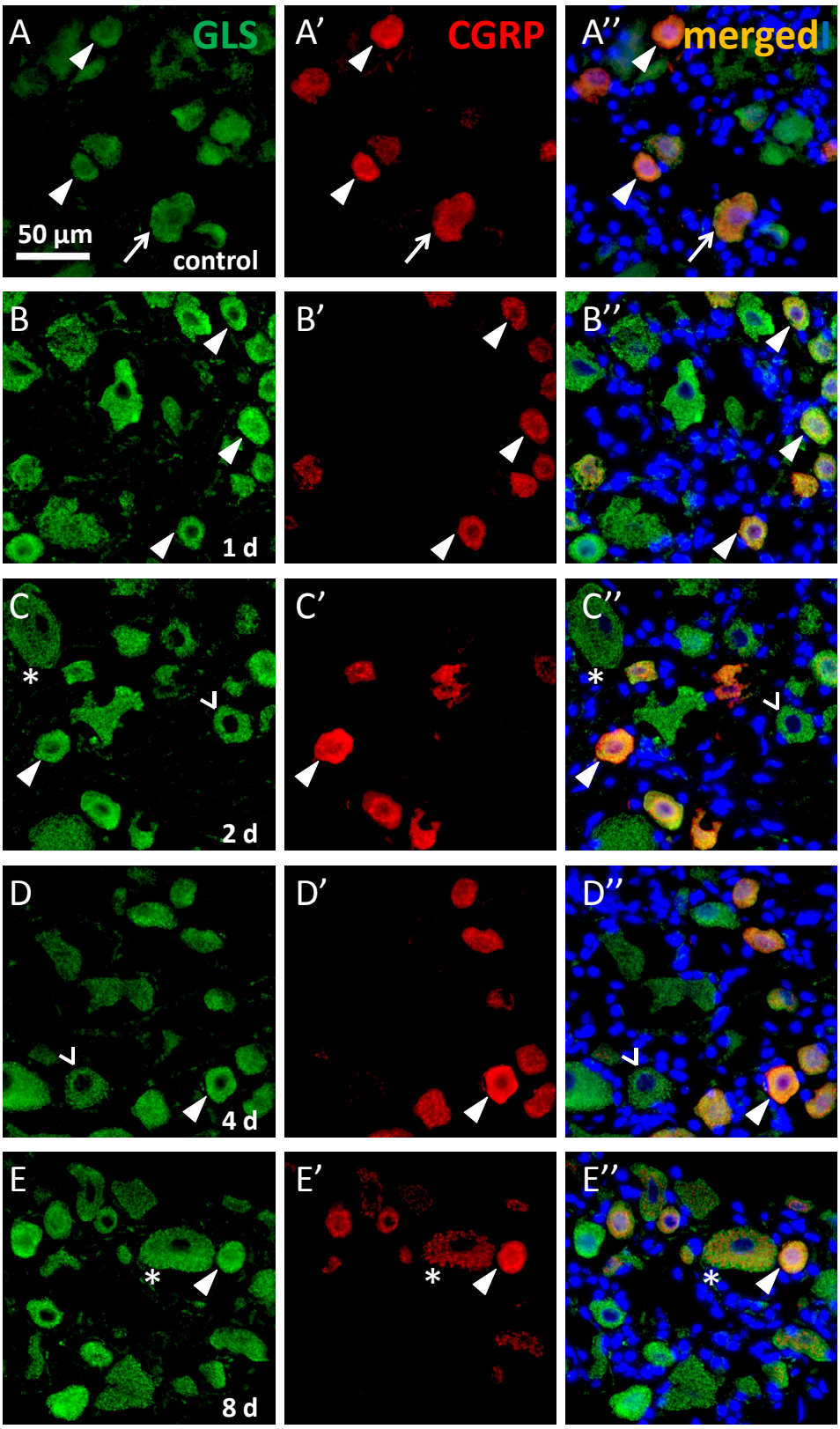

small
medium
large
100
80
60
40
20
0
SP-MGI
+ve
-ve
0
400
800
1200
1600
2000
2400
Area (μm2)

Figure 1. CGRP- **(A)** and SP- **(C)** MGI and the rank of individual DRG neuronal profiles (NPs) from naïve control animals (n=3) were pooled and plotted to reveal the change of pattern in MGI and to determine the MGI threshold for CGRP- and SP-ir. The rank of MGI was plotted on x-axis for easier observation. The MGI threshold was set at 10 and 20 for CGRP- and SP-MGI respectively based on the change in the shape and slope of the curve. NPs with CGRP-MGI equal or above 10 were considered as CGRP+ve, and NPs with SP-MGI equal or above 20 were considered as SP+ve. Scatter plot of CGRP- **(B)** and SP- **(D)** MGI against the cross-sectional area of individual NP. CGRP **(B)** had a higher proportion in naïve DRG (154 out of 387, 39.8%) compared to SP and was found across small (21.4%), medium (14.5%) and large (3.9%) DRG neurons. SP **(D)** has a smaller distribution in DRG (79 out 287, 27.5%) compared to CGRP. SP primarily labeled the small-sized neurons (20.9%) and a smaller proportion of medium-sized neurons (5.9%).

Figure 2



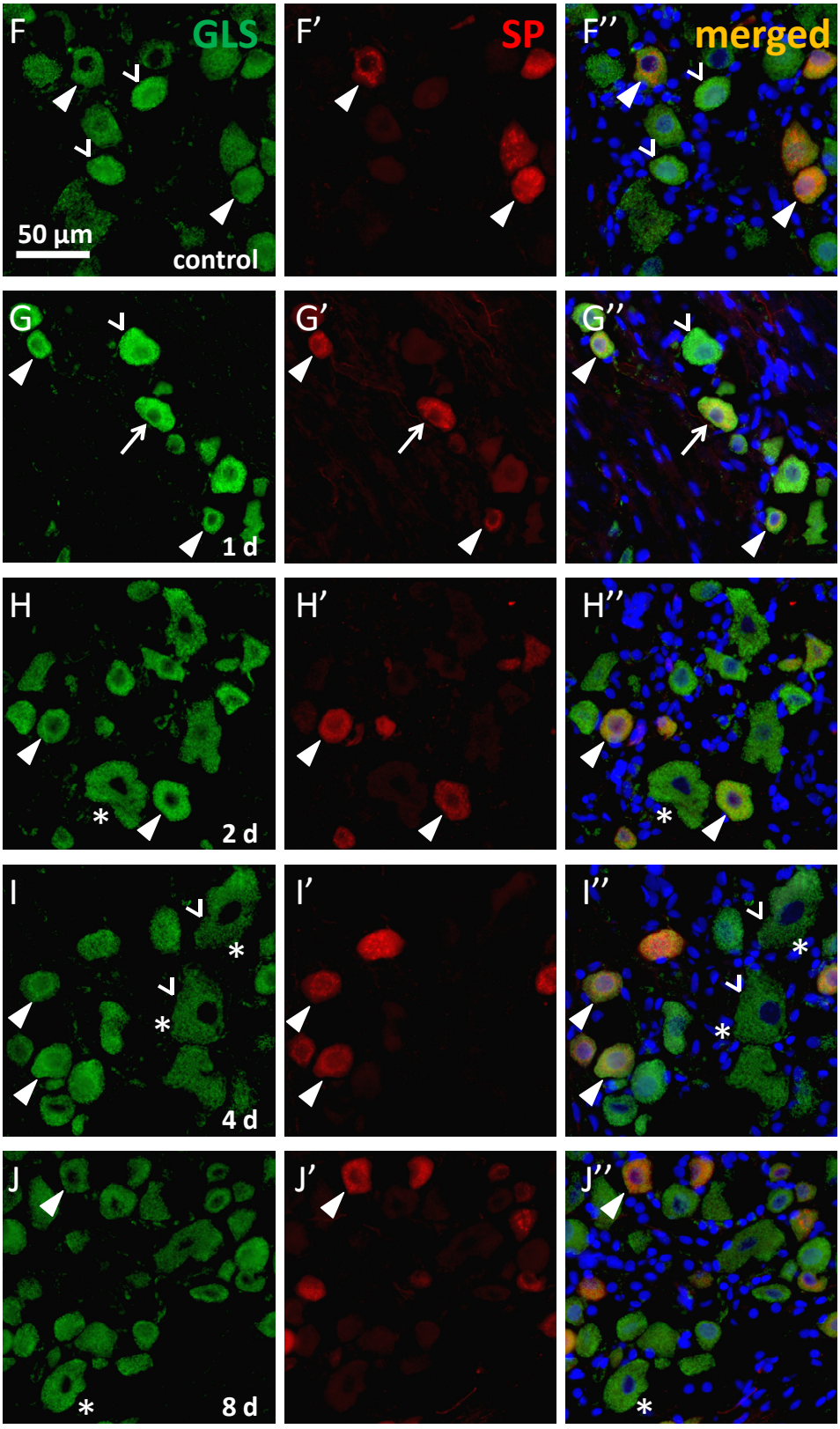
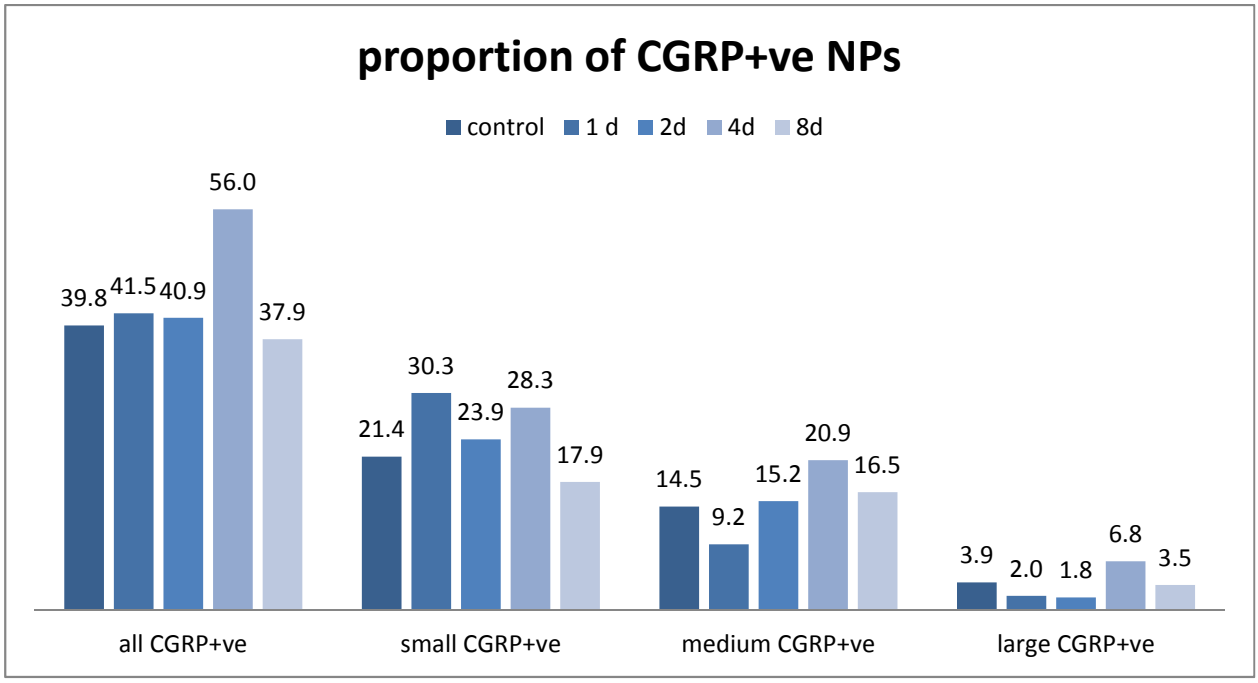

F
GLS
F'
SP
F''
merged
50 µm
control
G
G'
G''
1 d
H
H'
H''
2 d
I
I'
I''
4 d
J
J'
J''
8 d

Figure 2. Representative photomicrographs of DRG sections at different days following adjuvant-induced arthritis (AIA) and qualitative changes in immunoreactivity (ir) of GLS-neurons co-labeled with CGRP and SP. CGRP, SP and GLS have cytoplasmic staining in DRG sections. Note that CGRP- **(A'-E')** and SP-ir **(F'-J')** are found mainly in small- to medium-sized DRG neurons. GLS-ir **(A-J)** is in all DRG neurons, and no satellite cells or fibroblasts are stained positive for GLS-ir in naïve **(A, F)** or inflamed **(B-E, G-J)** animals. Neurons that express GLS with CGRP or SP appear yellow or orange in the merged images **(A''-J'')**. GLS-ir appears brighter in many DRG neurons at 1day AIA compared to control (A, B, F,G) and many are co-labeled with CGRP and SP (A", B", F", G"). Neuronal profiles (NPs) were counted with the following criteria: 1) the NP was through the center of the cell with a visible nucleus that was detected in DAPI filter; 2) the NP had an intact cytoplasm that did not touch the edge of the image. The cross-sectional area (μm^2) and mean gray intensity (MGI) of pixels in the cytoplasm (excluding the nucleus) was the recorded and measured as region of interest (ROI) with ImageJ for image analysis. (Arrowhead = small neurons, < 400 μm^2; arrow = medium neuron, 400-800 μm^2; * = large neuron, > 800 μm^2; open arrow = single-labeled neuron; blue = DAPI). Scale bar = 50 μm and the scale bar was applied to all the photomicrographs.

Figure 3

A.


proportion of CGRP+ve NPs
control
1 d
2d
4d
8d
39.8
41.5
40.9
56.0
37.9
21.4
30.3
23.9
28.3
17.9
14.5
9.2
15.2
20.9
16.5
3.9
2.0
1.8
6.8
3.5
all CGRP+ve
small CGRP+ve
medium CGRP+ve
large CGRP+ve

B.

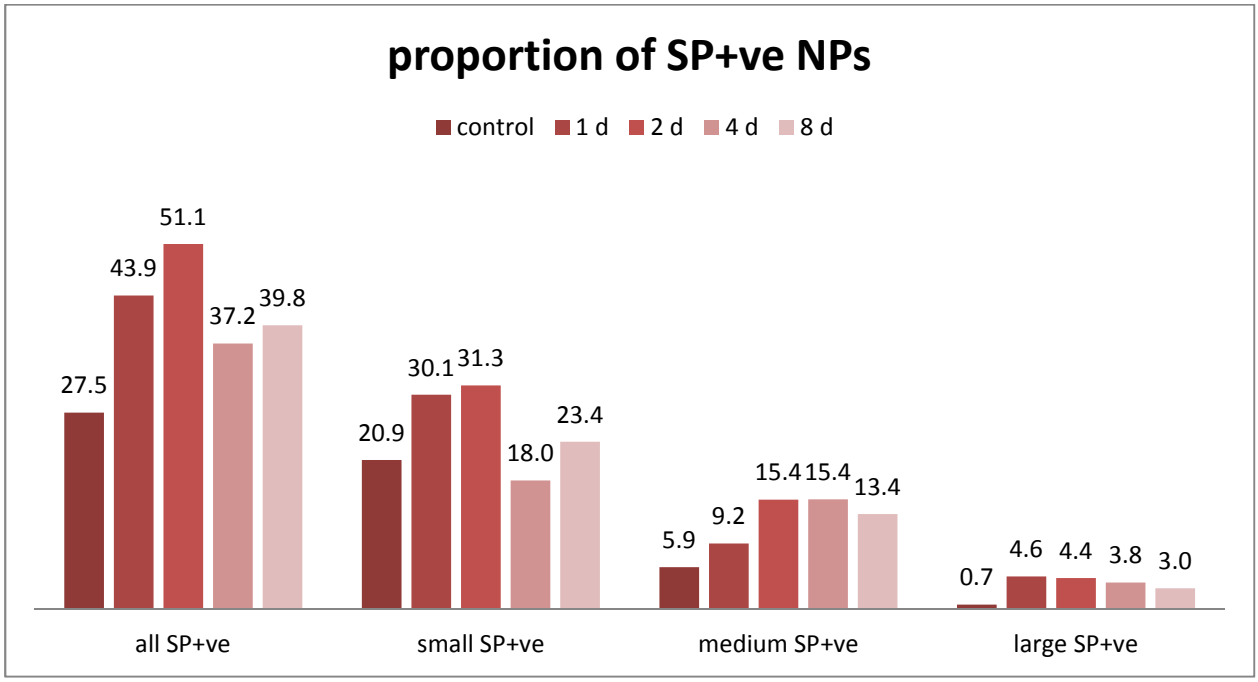

proportion of SP+ve NPs
control
1 d
2 d
4 d
8 d
27.5
43.9
51.1
37.2
39.8
20.9
30.1
31.3
18.0
23.4
5.9
9.2
15.4
15.4
13.4
0.7
4.6
4.4
3.8
3.0
all SP+ve
small SP+ve
medium SP+ve
large SP+ve

Figure 3. Temporal alteration of the proportion of CGRP+ve **(A)** and SP+ve **(B)** at different days during adjuvant-induced arthritis (AIA). **(A)** The maximum proportional increase of CGRP+ve neurons occurs at 4 days AIA (~16%), and small, medium and large neurons all contribute to the increased proportion at this time point. Note that at 1 day AIA, the proportion of small CGRP+ve neurons increases while that of medium CGRP+ve neurons decreases. **(B)** AIA produces an acute increase in the proportion of SP+ve neurons at 1 and 2 days and the increase is maintained through 8 days after AIA. Note that the proportion of medium (~10%) and large neurons significantly increased during the chronic phase of AIA (4-8 d).

Figure 4

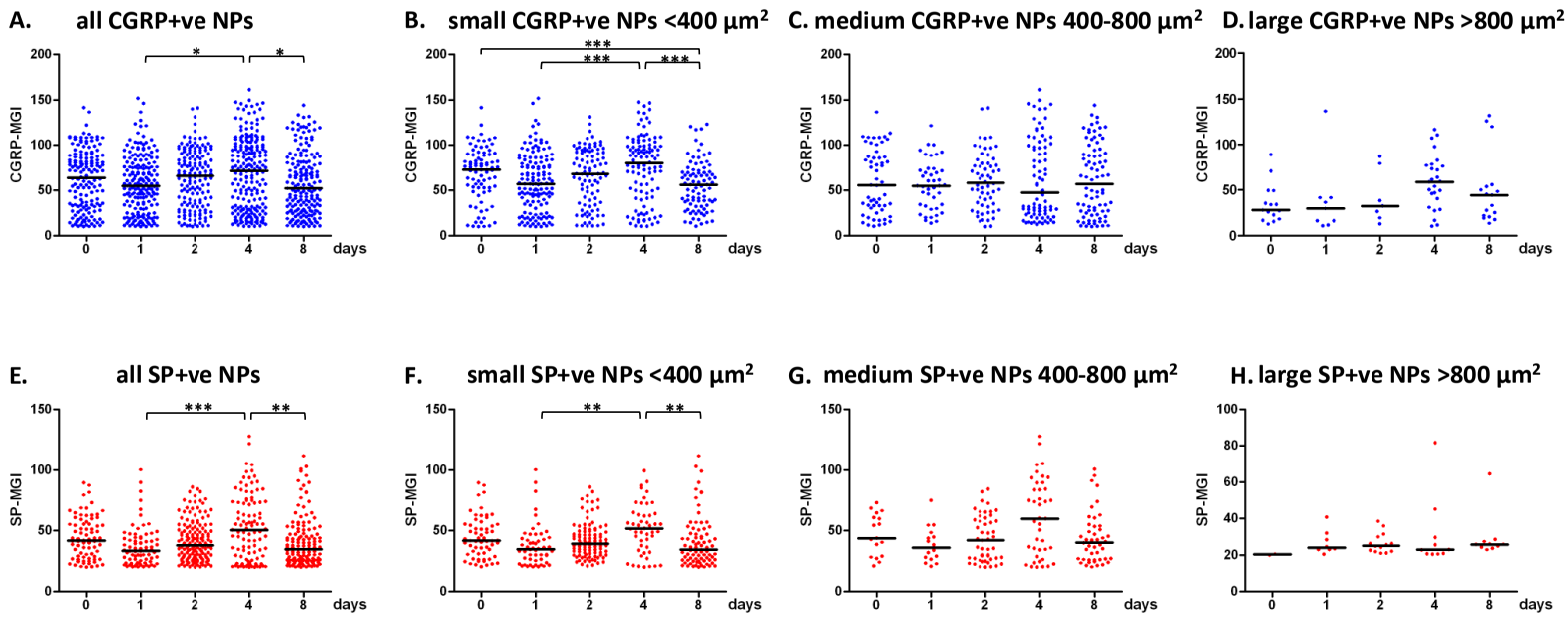

A. all CGRP+ve NPs
B. small CGRP+ve NPs <400 µm²
C. medium CGRP+ve NPs 400-800 µm²
D. large CGRP+ve NPs >800 µm²
CGRP-MGI
E. all SP+ve NPs
F. small SP+ve NPs <400 µm²
G. medium SP+ve NPs 400-800 µm²
H. large SP+ve NPs >800 µm²
SP-MGI
days
0
1
2
4
8
*
**
***

Figure 4. Temporal alteration of MGI in CGRP+ve **(A-D)** and SP+ve neurons **(E-F)** at different days during adjuvant-induced arthritis (AIA). When evaluating CGRP- and SP-MGI in all the traced neuronal profiles (NPs), CGRP- **(A)** and SP- **(E)** MGI at 4 days AIA is significantly elevated compared to that of 1 and 8 days AIA. No statistical difference is found at any day following AIA compared to the naïve animals in neither CGRP- nor SP-labeled NPs. When the NPs were further divided into small, medium and large sizes, the elevated CGRP- and SP-MGI occurred in small CGRP- **(B)** and SP- **(F)** containing NPs at 4 days after AIA, respectively. Kruskal-Wallis nonparametric one-way ANOVA was performed followed by Dunn posthoc test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 5

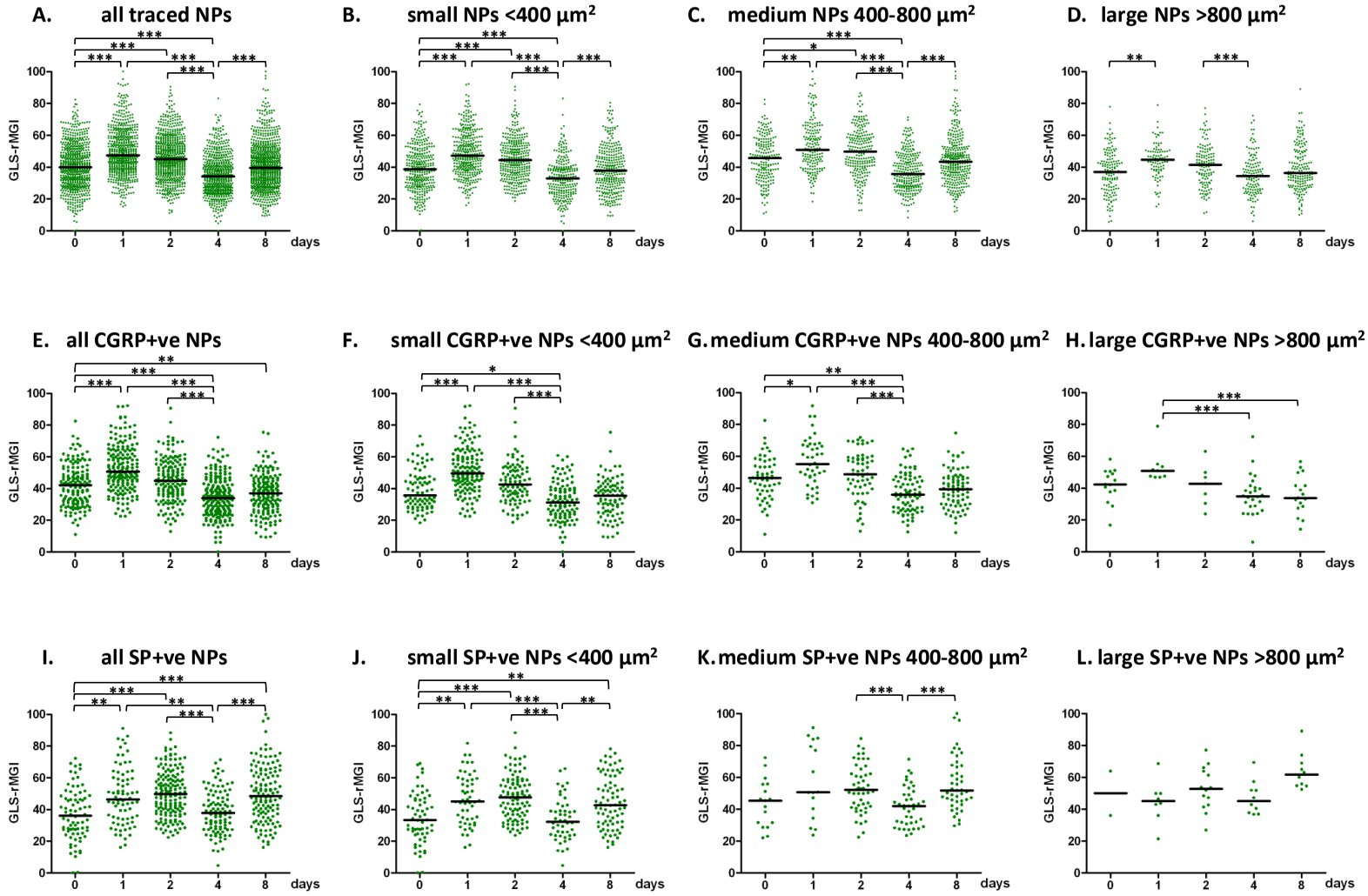

A. all traced NPs
B. small NPs <400 µm²
C. medium NPs 400-800 µm²
D. large NPs >800 µm²
E. all CGRP+ve NPs
F. small CGRP+ve NPs <400 µm²
G. medium CGRP+ve NPs 400-800 µm²
H. large CGRP+ve NPs >800 µm²
I. all SP+ve NPs
J. small SP+ve NPs <400 µm²
K. medium SP+ve NPs 400-800 µm²
L. large SP+ve NPs >800 µm²
GLS-rMGI
days
0
1
2
4
8

Figure 5. Temporal alteration of relative GLS-MGI (GLS-rMGI) in all traced neuronal profiles (NPs) **(A-D)**, CGRP+ve **(E-H)** and SP+ve **(I-L)** NPs at different days during adjuvant-induced arthritis (AIA). **(A-D)** In all the traced NPs **(A)**, an acute increase of GLS-rMGI occurs at 1 and 2 days AIA. GLS-rMGI drops below control level at 4 days AIA and restores to baseline at 8 days. In small- **(B)** and medium- **(C)** sized DRG NPs, the temporal alteration pattern is similar to that in all traced NPs **(A)**, while in large NPs, significant elevation of GLS-rMGI is only observed at 1 day following AIA **(D)**. **(E-H)** In all CGRP-labeled **(E)** DRG NPs, GLS showed a significant elevation at 1 day AIA, returning to baseline level at 2 days. At 4 and 8 days AIA, the GLS-rMGI stays below baseline level. A similar temporal pattern was observed in small- **(F)** and medium- **(G)** CGRP-labeled DRG NPs. In large CGRP-labeled NPs, GLS-rMGI at 4 and 8 days AIA significantly decreases compared to that of 1 day AIA **(H)**. **(I-L)** In all SP-labeled **(L)** DRG NPs, GLS-rMGI shows a biphasic elevation at both acute and chronic phases of AIA. The increase of GLS-rMGI starts immediately after AIA is initiated at 1 day and reaches maximum relative intensity at 2 days. From 2 to 4 days AIA, GLS-rMGI in all SP-labeled neurons shows a decrease that is below baseline and a second elevation occurs at 8 days AIA. When the NPs are further divided by size, a similar temporal alteration pattern is present in small SP-containing NPs **(J)**. In medium SP-labeled neurons, GLS-rMGI at 4 day AIA was significantly lower than those of 1 and 8 day AIA **(K)**. Note that in large CGRP+ve **(H)** and SP+ve **(L)** NPs, a similar temporal alteration pattern of GLS-rMGI appears to occur as in all traced NPs **(E)** and **(I**but without statistical difference (possibly due to the limited number of large NPs labeled by the two neuropeptides). This analysis indicates that peripheral inflammation may cause change in expression of GLS in all size classes of DRG neurons. Kruskal-Wallis nonparametric one-way ANOVA was performed followed by Dunn posthoc test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

CHAPTER III

INCREASED ACCUMULATION OF GLUTAMINASE, CALCITONIN GENE-RELATED PEPTIDE, AND SUBSTANCE P IN RAT SCIATIC NERVE FOLLOWING ADJUVANT-INDUCED ARTHRITIS

Abstract

Increased nociceptive transmission is one of the major symptoms in both acute and chronic inflammation. As a neurotransmitter, glutamate has an important role in nociceptive transmission, and elevated glutamate levels have been reported at the peripheral terminal of primary sensory neurons following peripheral inflammation. Glutaminase (GLS) is the synthetic enzyme for glutamate production in dorsal root ganglion (DRG) neurons. Elevated glutaminase expression and enzymatic activity have been described in DRG neuronal cell bodies within 1 day of inflammation and lasting up to 8 days. In addition, elevated amounts of glutaminase have been described in peripheral nerve terminals at the inflamed site during the chronic phase of adjuvant-induced arthritis (AIA). In the current study, therefore, glutaminase level was evaluated within the sciatic nerve during AIA. It was accomplished by examining the accumulation of glutaminase, after the initiation of AIA, in the sciatic nerve proximal to a ligature. Calcitonin gene-related peptide (CGRP) and substance P (SP) were also investigated as two putative neuropeptides produced by nociceptive neurons. Increased accumulation of GLS, CGRP and SP immunoreactivity (-ir) was found at the proximal side of the ligature in response to AIA. The increased glutaminase transported in the sciatic nerve may promote increased glutamate

production and release at the peripheral terminal, leading to additional activation of peripheral glutamate receptors and contribution to the development, modification and maintenance of inflammatory pain.

Introduction

In primary sensory neurons, glutamate has an important role in the initiation, modulation and maintenance of nociceptive transmission. Elevated glutamate levels have been reported at the peripheral nerve terminal contributing to the hypersensitivity during acute and chronic inflammation (Du et al., 2001, Cairns et al., 2002, Beirith et al., 2003, Aumeerally et al., 2004). The increased glutamate release that rapidly occurs in acute tissue inflammation may come from depletion of glutamate-containing synaptic vesicles and stimulation of local glutamate production via its synthetic enzyme glutaminase at the nerve terminals in response to inflammation (deGroot et al., 2000, Medvedeva et al., 2008, Jin et al., 2009). For the elevated glutamate levels in peripheral tissue in some chronic pain models, a long-term mechanism that leads to elevated glutamate production is expected. In the central and peripheral nervous system, glutaminase is the major synthetic enzyme that replenishes the neurotransmitter pool for glutamate release (Hamberger et al., 1979, Bradford et al., 1989, Kvamme et al., 2001). Studies using experimental cerebral ischemia, Alzheimer's disease (AD) and HIV-associated neurocognitive disease (HAND) and chronic inflammatory pain demonstrate that increased glutaminase concentration and enzyme activity contribute to elevated glutamate production (Newcomb et al., 1997, Newcomb et al., 1998, Weng et al., 2006, Miller et al., 2010, Miller et al., 2012). In Chapter II, the results showed that there is a temporal alteration of glutaminase, the major synthetic enzyme of glutamate for replenishing the neurotransmitter pool, in response to adjuvant-induced arthritis (AIA) in the neuronal cell bodies located in dorsal root ganglion (DRG). An acute elevation of GLS expression was observed in the DRG neuronal cell bodies at 24h and 48h, followed by a gradual decrease at 4 days after initiation of AIA. At 7 days following AIA, elevated glutaminase

expression is found in the inflamed hindpaw skin (Miller et al., 2012). If elevated glutaminase production occurs in DRG neuronal cell bodies in response to inflammation and if there is elevated glutaminase found in the hindpaw, we hypothesized that altered glutaminase levels could be detected in the sciatic nerve at specific time points during AIA. To address this issue, sciatic nerve ligation at different time intervals after initiation of inflammation was used to halt axonal transport and study the accumulation of substances transported from the neuronal cell bodies of the DRG.

Materials and methods

Male Sprague-Dawley rats (200 – 300g) were housed in a 12:12 light/dark cycle and given access to food and water *ad libitum*. Rats were anesthetized with isoflurane (5% induction, 2% maintenance) before any injection or surgery. Adjuvant-induced arthritis (AIA) was induced by a single unilateral subcutaneous injection of 150 µl of 1:1 of saline/CFA emulsion (complete Freund's adjuvant, 150 µg/150 µl) in the hindpaw plantar surface. AIA was allowed to develop for four, three, two, and one days prior to sciatic nerve ligation surgery. On the day of surgery, rats were anesthetized and the ispsilateral sciatic nerve was tied at mid-thigh level with 4.0-silk suture (Ethicon, Ethicon Inc.). The same surgery was performed on naïve rats with no injection as a control. After the surgery, the rats were left in new cages for 24 hours to allow for accumulation of protein at the ligature. Procedures were carried out in accordance with the National Institute of Health Guide for the care and use of laboratory animals and were approved by the committee of animal care and use for research at Oklahoma State University Center for Health Sciences. All efforts were made to minimize the number of animals used and their suffering.

In the present study, all tissue collections were performed on the same day of the experiment and processed for immunohistochemistry under the same conditions to minimize the batch-to-batch variations introduced during tissue handling and processing. Rats were anesthetized with 3 ml of Avertin (2.5% of 2, 2, 2-Tribromoethanol, Sigma-Aldrich) and 0.8 ml of xylazine (1 mg/ml,

AnaSed, LLOYD). The rats were perfused with 80 ml calcium free Tyrode's solution followed by 300 ml optimized fixative for GLS immunolabeling described in previous papers (Hoffman et al., 2010). Briefly, it contains 0.2% (w/v) paraformaldehyde, 70% (v/v) picric acid, 0.1M sodium phosphate buffer at pH 7.3. The ipsilateral sciatic nerve was removed from each rat with unequal lengths in relation to the ligature to identify the proximal and distal sides. The nerve segments were post-fixed in the same fixative for 4 h at 4°C. Tissues were transferred to 10% sucrose in PBS, pH 7.3 overnight at 4°C and frozen sections were cut at 14 μm with a HM550 cryostat (Thermo Scientific). Sections were affixed to gelatin-coated glass microscope slides and every fifth section was used per antiserum. Dried sections were rinsed three times with PBS and blocked in 0.5% (w/v) polyvinlypyrolidone and 0.5% bovine serum albumin in PBS with 0.3% (v/v) Triton X-100 (PBS-T, Sigma). The polyclonal rabbit anti-glutaminase antiserum was a generous gift from Dr. Norman Curthoys (Colorado State University). Sections were incubated in the antisera containing rabbit-anti glutaminase (1:20,000) with mouse anti-CGRP (1:8,000; Santa Cruz) for four days at 4°C as described previously (Hoffman et al., 2010). After incubation in primary antisera, sections were rinsed three times in PBS and incubated in secondary antisera containing biotinylated goat anti-rabbit IgG (1.0 μg/ml; Vector Laboratories; Burlingame, CA, USA) and Alexa Flour 555 conjugate goat anti-mouse IgG (1.67 μg/ml; Invitrogen; Carlsbad, CA, USA) diluted in PBS-T for 1 h at room temperature. Sections were rinsed twice in PBS and once in sodium carbonate buffered saline (SCBS, pH 9.6) before incubating for 1 h in 1.0 μg/ml avidin-fluorescein isothiocyanate (FITC; Vector Laboratories) diluted in SCBS. After three PBS rinses, coverslips were apposed to slides with ProLong Gold Mouting Media (Invitrogen). Additional sections were processed singly in mouse anti-SP (1:2,000; R&D Systems) and followed by Alexa Flour 555 conjugate goat anti-mouse IgG using a similar protocol. Epifluoresence images were photographed with a 20X objective with an Olympus BX51 microscope (Olympus; Center Valley, PA, USA) using a SPOT RT740 camera (Diagnostic Instruments; Sterling Heights, MI, USA). The micrographs were stored as 8-bit grayscale tiff

format with the pixel intensity value ranging from 0-255. Analysis was performed with Image J software (National Institutes of Health; Bethesda, MD, USA). For sciatic nerve analysis, image analysis was adapted from previous studies (Long et al., 1998, Zhang et al., 2000, Thakor et al., 2009). Briefly, five 50×50 μm^2 squares were drawn within a distance of P0 (0-200 μm) and P1 (200-400 μm) respectively from the proximal side of the ligature. Images were excluded that contained gaps, breaks, or signal artifact. An average accumulation (P) of P0 and P1 was also calculated to reveal the total accumulation of substances at the proximal side of the ligature. Only the fluorescent staining within nerve tissue was included in the measurements. The data of the five sections was averaged to give a mean gray value. This method of analysis permits quantification of the relative mean gray value of immunoreactive staining for comparison among the different AIA time points. The data was expressed as Mean ± S.E.M. Significant differences in MGI among different experimental conditions were determined by the Kruskal-Wallis one-way ANOVA by ranks followed by *Dunn posthoc* test (Prism version 5.03, GraphPad Software Inc., La Jolla, CA). In all analyses, *P*-value less than 0.05 were considered significant. * $P< 0.05$, ** $P< 0.01$, *** $P< 0.001$.

Results

Figure 1 shows the appearance of the proximal side of a ligated peripheral nerve. Immunoreactivity (-ir) of glutaminase, calcitonin gene-related peptide (CGRP) and substance P (SP) was visualized. The fluorescent intensity was more intense around the region that was immediately proximal to the ligature. The proximal side was further divided as P0 (within 200μm of the tie, Fig. 2A, 2D and 2G) and P1 (200-400μm from the tie, Fig. 2B, 2E and 2H). An average accumulation P (Fig. 2C, 2F and 2I) was defined as the mean of P0 and P1.

The immunoreactivity (-ir) of glutaminase at the site of ligature of sciatic nerve was compared from naïve sham control to 1-4 days of AIA by quantifying the mean gray intensity (MGI) from

image analysis. Elevated glutaminase-ir in section P0 was observed at 1 d (~36%), 2 d (~60%, $p < 0.01$), 3 d (~19%) and 4 d (~15%) following AIA compared to naïve sham control (Fig. 2A). No significant elevated glutaminase-ir was observed in section P1 (Fig. 2B). The average increased accumulation P of glutaminae-ir was observed at 1 d (~29%), 2 d (~58%, $p < 0.01$), 3 d (~12%) and 4 d (~12%) of AIA compared to naïve control (Fig. 2C). The peak glutaminase accumulation occurred at 2 d of AIA.

A similar pattern and peak accumulation at 2 day AIA was observed in CGRP-ir. Elevated CGRP-ir in section P0 was observed at 1 d (~48%), 2 d (~82%, $p < 0.01$), 3 d (~42%) and 4 d (~38%) following AIA compared to naïve sham control (Fig. 2D). Elevated CGRP-ir in section P1 was also observed at 1 d (~54%), 2 d (~151%), 3 d (~64%) and 4 d (~24%) following AIA compared to naïve sham control (Fig. 2D), but no statistical significance was achieved in section P1 (Fig. 2E). The average increased accumulation P of CGRP-ir was observed at 1 d (~50%), 2 d (~110%, $p < 0.01$), 3 d (~51%) and 4 d (~32%) of AIA compared to naïve control (Fig. 2F).

The temporal accumulation of SP occurred with a different pattern compared to that of glutaminase and CGRP. Elevated SP-ir in section P0 was observed at 1 d (~112%, $p < 0.001$), 2 d (~133%, $p < 0.001$), 3 d (~112%, $p < 0.001$) and 4 d (~99%, $p < 0.001$) following AIA compared to naïve sham control (Fig. 2G). Elevated SP-ir in section P1 was also observed at 1 d (~181%, $p < 0.001$), 2 d (~155%, $p < 0.001$), 3 d (~108%, $p < 0.001$) and 4 d (~199%, $p < 0.001$) following AIA compared to naïve sham control (Fig. 2H). The average increased accumulation P of SP-ir was observed at 1 d (~134%, $p < 0.001$), 2 d (~140%, $p < 0.001$), 3 d (~111%, $p < 0.001$) and 4 d (~130%, $p < 0.001$) of AIA compared to naïve control (Fig. 2I).

Discussion

In previous studies, it was found that adjuvant-induced arthritis (AIA) causes elevated glutaminase expression in rat DRG neuronal cell bodies during both the acute and chronic phases

of inflammation (Miller et al., 2012; Zhang, Chapter 2). The acute elevation occurred from 24 h to 48 h, followed by a decrease below basal level at 4 d. At 7 days following AIA, elevated glutaminase expression is found in the inflamed hindpaw skin (Miller, 2007, Hoffman, 2009, Miller et al., 2010). The temporal elevation in the neuronal cell bodies and their peripheral target tissue during different time points of AIA raises the question: Can increased levels of glutaminase be detected in the peripheral nerve following inflammation? In order to answer this question, the accumulation of glutaminase at proximal side of a ligature of the sciatic nerve was monitored during different intervals of peripheral inflammation. Calcitonin gene-related peptide and substance P were evaluated, also. These results showed that there is increased accumulation of glutaminase, CGRP and SP at the proximal side of the ligature at 2 days following AIA. These results show that elevated glutaminase and neuropeptides occur in the peripheral nerve following production in the DRG neuronal cell bodies in response to inflammation.

In the current study, naïve rats with ligatures were included as the control to manifest the accumulation at basal levels since the basal transport of glutaminase and neuropeptides have a low detection level in nerve without ligation in our pilot studies (unpublished observation). This has been a common approach to evaluate organelle and protein transport (Zelena et al., 1968, McDougal et al., 1981, Kashihara et al., 1989, Smith et al., 1992), but ligatures constraining the nerve are a type of nerve injury (Wall and Devor, 1981, Ma and Bisby, 1998). The possibility exists that the accumulation of proteins is due to local nerve inflammation and/or nerve injury. An unequal accumulation of proteins, however, was found at different time points post CFA-injection with the same 24 hour ligature at all time points compared to the sham-naïve control group (Fig. 1 and Fig. 2). This provides comparison among the different time intervals from the onset of inflammation. These results indicate that the accumulation of increased glutaminase, CGRP and SP in peripheral nerve occurs with a temporal pattern consistent with the development

of inflammation and the influence of inflammation on protein production in DRG neuronal cell bodies.

In primary afferents, many substances that have important neuronal function are not restricted to the neuronal cell bodies. They are delivered to the nerve terminal or their active site outside neuronal cell bodies by axonal transport. For example, neuropeptides, such as CGRP and SP, are synthesized in neuronal cell bodies. After sorting and packaging into large dense-core vesicles (LDCVs), the neuropeptides are transported to the nerve terminal and released in response to nerve terminal activation. Increased concentration of CGRP and SP has been observed in the neuronal cell bodies (Zhang, Chapter II) and peripheral branches of primary afferents following inflammation (Donnerer et al., 1992, Nahin and Byers, 1994). These results are in accordance with previous findings indicating that peripheral inflammation up-regulates the contents and transport of CGRP and SP in sensory nerves from neuronal cell bodies.

Studies on brain/kidney GLS show that there are two major sub-cellular locations of GLS, a cytoplasmic GLS and mitochondrial GLS (Zhang, Schechter, Miller, unpublished observations). Under normal conditions, GLS is synthesized in the neuronal cytoplasm and transported to the outer face of the mitochondrial inner membrane (Kvamme et al., 2000, Kvamme et al., 2001). The mitochondrial GLS is considered to be the active site of glutamate production and the cytoplasmic GLS is inactive due to the high cytosolic concentration of glutamate (Kvamme et al., 2000, Miller et al., 2012). Mitochondria are one of the organelles that are in motion in the axoplasm and GLS enzyme activity is found accumulated at the proximal side of sciatic nerve ligature (McDougal et al., 1981, McDougal et al., 1983). Studies using an *in vitro* culture system showed that mitochondria accumulate at the site of NGF stimulation. This was performed by stimulating cultured axons with NGF-coated beads at points distant from their cell bodies or growth cones (Chada and Hollenbeck, 2003, 2004). Although the transport machinery of GLS in the peripheral axons has not been identified, these studies and the present results support the

hypothesis that elevated GLS can be mobilized and transported by mitochondria in peripheral nerve axons to their nerve terminals where glutamate synthesis occurs for replenishing the neurotransmitter pool. The current results indicate that the peripheral transport of altered levels of glutaminase occurs temporally in accordance the alteration pattern of glutaminase observed in the neuronal cell bodies (Zhang, Chapter II). This peripherally transported glutaminase is likely to arrive at peripheral terminal during the chronic phase of inflammation. Elevated glutaminase concentration and activity found in the inflamed skin may be newly transported from the neuronal cell bodies and this can be one of the mechanisms that contributes to elevated glutamate production at the peripheral terminal in chronic inflammatory pain (Miller et al., 2010).

These results characterize the axoplasmic transport of glutaminase in peripheral nerve at different time points during the rat AIA model. Elevated glutaminase immunoreactivity may indicate an anterograde transport of GLS from the DRG neuronal cell body to the inflamed tissue. This would be the most direct evidence that the elevated glutaminase in DRG after AIA is transported to peripheral tissue. The elevated levels shipped by peripheral transport increase likelihood of increased glutamate synthesis and peripheral release. Augmented glutamate release would increase activation of excitatory amino acid receptors on peripheral nerve terminals enhancing the generation, modification and maintenance of nociceptive transmission.

References

Aumeerally N, Allen G, Sawynok J (2004) Glutamate-evoked release of adenosine and regulation of peripheral nociception. Neuroscience 127:1-11.

Beirith A, Santos AR, Calixto JB (2003) The role of neuropeptides and capsaicin-sensitive fibres in glutamate-induced nociception and paw oedema in mice. Brain research 969:110-116.

Bradford HF, Ward HK, Foley P (1989) Glutaminase inhibition and the release of neurotransmitter glutamate from synaptosomes. Brain research 476:29-34.

Cairns BE, Gambarota G, Svensson P, Arendt-Nielsen L, Berde CB (2002) Glutamate-induced sensitization of rat masseter muscle fibers. Neuroscience 109:389-399.

Chada SR, Hollenbeck PJ (2003) Mitochondrial movement and positioning in axons: the role of growth factor signaling. The Journal of experimental biology 206:1985-1992.

Chada SR, Hollenbeck PJ (2004) Nerve growth factor signaling regulates motility and docking of axonal mitochondria. Current biology : CB 14:1272-1276.

deGroot J, Zhou S, Carlton SM (2000) Peripheral glutamate release in the hindpaw following low and high intensity sciatic stimulation. Neuroreport 11:497-502.
Donnerer J, Schuligoi R, Stein C (1992) Increased content and transport of substance P and calcitonin gene-related peptide in sensory nerves innervating inflamed tissue: evidence for a regulatory function of nerve growth factor in vivo. Neuroscience 49:693-698.
Du J, Koltzenburg M, Carlton SM (2001) Glutamate-induced excitation and sensitization of nociceptors in rat glabrous skin. Pain 89:187-198.
Hamberger A, Chiang GH, Sandoval E, Cotman CW (1979) Glutamate as a CNS transmitter. II. Regulation of synthesis in the releasable pool. Brain research 168:531-541.
Hoffman EM (2009) The role of dorsal root ganglion glutaminase in nociception - A novel therapeutic target for inflammatory pain. In: Ann Arbor: ProQuest/UMI.
Hoffman EM, Schechter R, Miller KE (2010) Fixative composition alters distributions of immunoreactivity for glutaminase and two markers of nociceptive neurons, Nav1.8 and TRPV1, in the rat dorsal root ganglion. J Histochem Cytochem 58:329-344.
Jin YH, Yamaki F, Takemura M, Koike Y, Furuyama A, Yonehara N (2009) Capsaicin-induced glutamate release is implicated in nociceptive processing through activation of ionotropic glutamate receptors and group I metabotropic glutamate receptor in primary afferent fibers. Journal of pharmacological sciences 109:233-241.
Kashihara Y, Sakaguchi M, Kuno M (1989) Axonal transport and distribution of endogenous calcitonin gene-related peptide in rat peripheral nerve. The Journal of neuroscience : the official journal of the Society for Neuroscience 9:3796-3802.
Kvamme E, Roberg B, Torgner IA (2000) Phosphate-activated glutaminase and mitochondrial glutamine transport in the brain. Neurochemical research 25:1407-1419.
Kvamme E, Torgner IA, Roberg B (2001) Kinetics and localization of brain phosphate activated glutaminase. Journal of neuroscience research 66:951-958.
Long A, Bongenhielm U, Boissonade FM, Fried K, Robinson PP (1998) Neuropeptide immunoreactivity in ligature-induced neuromas of the inferior alveolar nerve in the ferret. Brain research 791:263-270.
Ma W, Bisby MA (1998) Increase of preprotachykinin mRNA and substance P immunoreactivity in spared dorsal root ganglion neurons following partial sciatic nerve injury. The European journal of neuroscience 10:2388-2399.
McDougal DB, Jr., Yu MJ, Gorin PD, Johnson EM, Jr. (1981) Transported enzymes in sciatic nerve and sensory ganglia of rats exposed to maternal antibodies against nerve growth factor. Journal of neurochemistry 36:1847-1852.
McDougal DB, Jr., Yuan MJ, Dargar RV, Johnson EM, Jr. (1983) Neonatal capsaicin and guanethidine and axonally transported organelle-specific enzymes in sciatic nerve and in sympathetic and dorsal root ganglia. The Journal of neuroscience : the official journal of the Society for Neuroscience 3:124-132.
Medvedeva YV, Kim MS, Usachev YM (2008) Mechanisms of prolonged presynaptic Ca2+ signaling and glutamate release induced by TRPV1 activation in rat sensory neurons. The Journal of neuroscience : the official journal of the Society for Neuroscience 28:5295-5311.
Miller KE (2007) Method of alleviating chronic pain via peripheral glutaminase regulation. vol. 7,288,246 B2 United States: The Board of Regents of the University of Oklahoma.
Miller KE, Balbas JC, Benton RL, Lam TS, Edwards KM, Kriebel RM, Schechter R (2012) Glutaminase immunoreactivity and enzyme activity is increased in the rat dorsal root ganglion following peripheral inflammation. Pain Res Treat 2012:414697.
Miller KE, Herzog B, Sutharshan M (2010) 6-Diazo-5-Oxo-L-Norleucine: A Glutaminase Inhibitor with Local Analgesic Properties. In: IASP, vol. 4193 Montreal, Qeubec, Canada.

Nahin RL, Byers MR (1994) Adjuvant-induced inflammation of rat paw is associated with altered calcitonin gene-related peptide immunoreactivity within cell bodies and peripheral endings of primary afferent neurons. The Journal of comparative neurology 349:475-485.

Newcomb R, Pierce AR, Kano T, Meng W, Bosque-Hamilton P, Taylor L, Curthoys N, Lo EH (1998) Characterization of mitochondrial glutaminase and amino acids at prolonged times after experimental focal cerebral ischemia. Brain research 813:103-111.

Newcomb R, Sun X, Taylor L, Curthoys N, Giffard RG (1997) Increased production of extracellular glutamate by the mitochondrial glutaminase following neuronal death. The Journal of biological chemistry 272:11276-11282.

Smith GD, Harmar AJ, McQueen DS, Seckl JR (1992) Increase in substance P and CGRP, but not somatostatin content of innervating dorsal root ganglia in adjuvant monoarthritis in the rat. Neuroscience letters 137:257-260.

Thakor DK, Lin A, Matsuka Y, Meyer EM, Ruangsri S, Nishimura I, Spigelman I (2009) Increased peripheral nerve excitability and local NaV1.8 mRNA up-regulation in painful neuropathy. Molecular pain 5:14.

Wall PD, Devor M (1981) The effect of peripheral nerve injury on dorsal root potentials and on transmission of afferent signals into the spinal cord. Brain research 209:95-111.

Weng HR, Chen JH, Cata JP (2006) Inhibition of glutamate uptake in the spinal cord induces hyperalgesia and increased responses of spinal dorsal horn neurons to peripheral afferent stimulation. Neuroscience 138:1351-1360.

Zelena J, Lubinska L, Gutmann E (1968) Accumulation of organelles at the ends of interrupted axons. Z Zellforsch Mikrosk Anat 91:200-219.

Zhang JY, Luo XG, Xian CJ, Liu ZH, Zhou XF (2000) Endogenous BDNF is required for myelination and regeneration of injured sciatic nerve in rodents. The European journal of neuroscience 12:4171-4180.

Figure 1

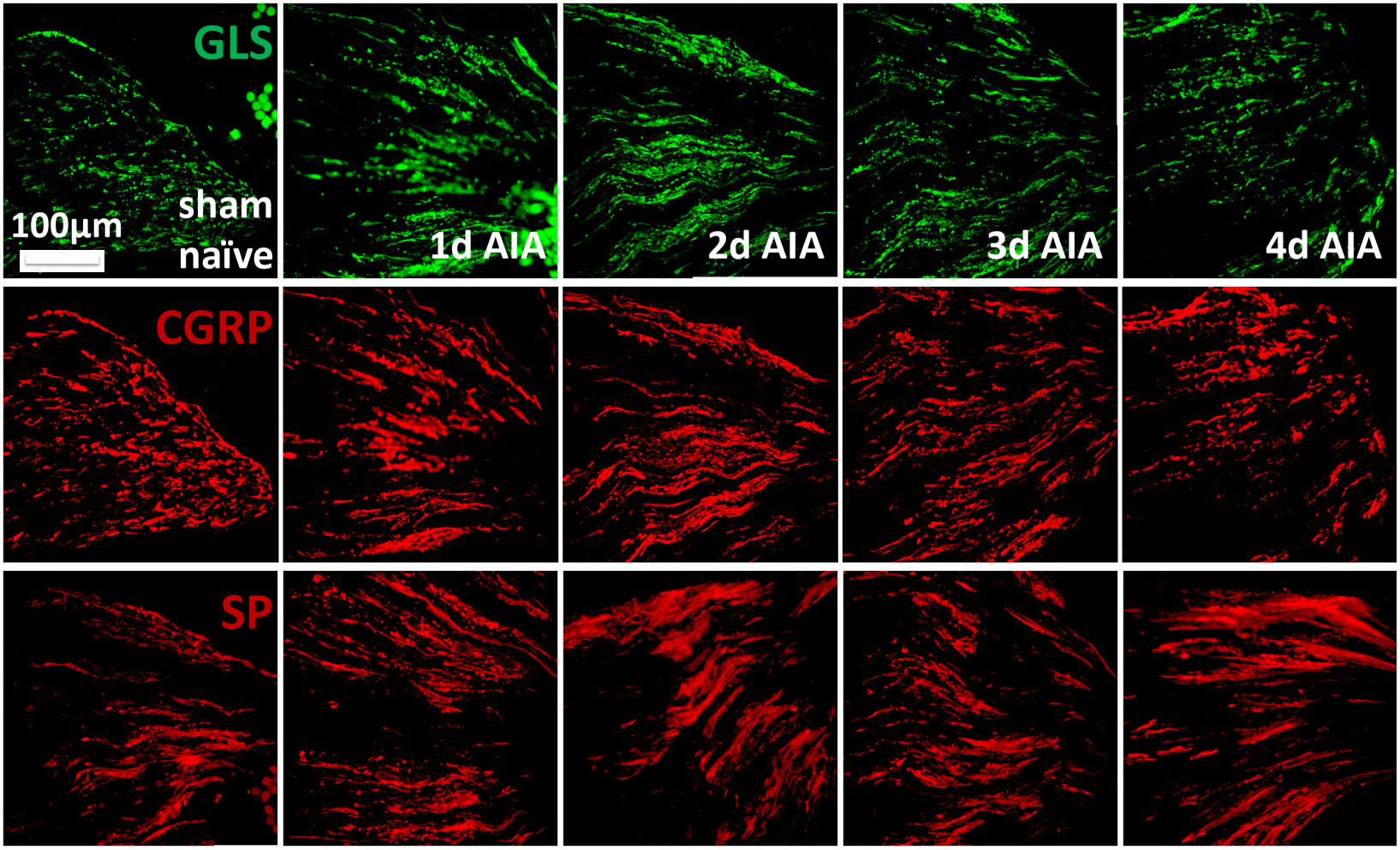
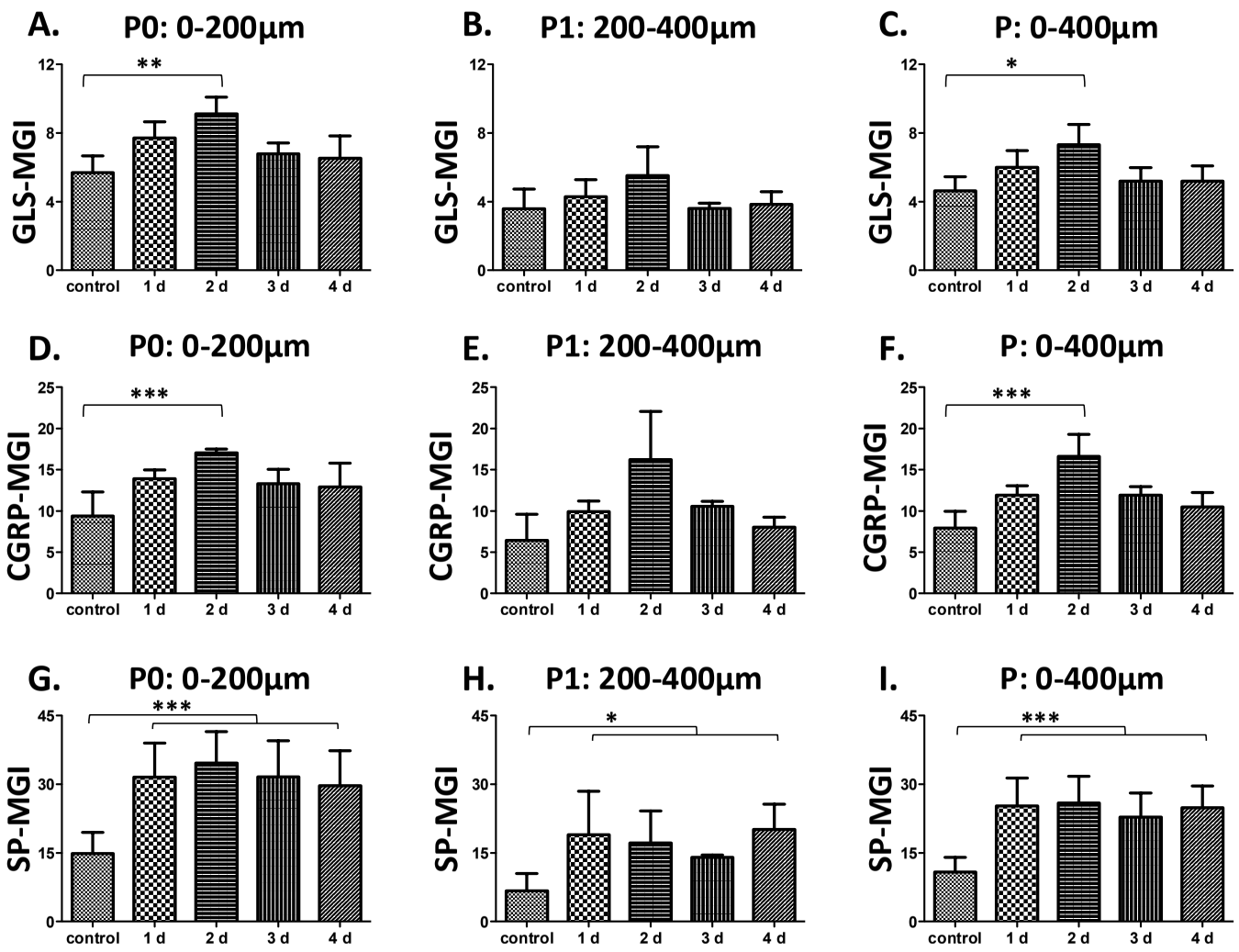


Figure 1. Photomicrographs showing substance accumulation near the proximal side of the ligature at different days following adjuvant-induced arthritis (AIA). The dorsal root ganglion is to the left and the peripheral target (hindpaw) located to the right side of the nerve segment. Glutaminase (GLS, green) are colocalized with calcitonin gene-related peptide (CGRP, red) and visualized via different filters. Single-labled substance P (SP, red) is also shown. A gradual narrowed region is formed by the ligature. Intensely stained nerve bundles located immediate to the tip region of the tie, which indicated the accumulation of substances by disrupting the axonal transport. A qualitative increase in substance accumulation was observed at 2 days following AIA for GLS, CGRP and SP. Scale bar is set at 100 μm and is applied to all photomicrographs

Figure 2


A.
P0: 0-200µm
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H.
P1: 200-400µm
I.
P: 0-400µm
GLS-MGI
CGRP-MGI
SP-MGI
control
1 d
2 d
3 d
4 d
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Figure 2. Quantitative results of the temporal accumulation of GLS, CGRP and SP at the proximal side to the ligature in sciatic nerve following adjuvant-induced arthritis (AIA). **A-C**, the accumulation of immunoreactivity (-ir) of GLS was more prominent at P0 region compared to P1. In P0 region, elevated GLS-ir at 2 day of AIA was significantly higher compared to naïve-sham control (~ 60%, $p < 0.01$), and the average accumulation P increased by 58% ($p < 0.01$). **D-F**, the accumulation of CGRP was observed at region P0 (~ 82%, $p < 0.01$) at 2 days of AIA compared to sham control. **G-I**, a rapid two-fold accumulation is observed in SP-ir from 1 day of AIA and maintained till 4 day of AIA at both P0 and P1 region. Kruskal-Wallis nonparametric one-way ANOVA was performed followed by Dunn *posthoc* test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

CHAPTER IV

PERIPHERAL INHIBITION OF GLUTAMATE PRODUCTION AT THE ONSET OF ADJUVANT-INDUCED ARTHRITIS HAS ANTI-INFLAMMATORY EFFECTS AND REDUCES ELEVATION OF GLUTAMINASE IN THE RAT PEPTIDE-CONTAINING DORSAL ROOT GANGLION NEURONS

Abstract

Glutamate, released from peripheral afferents, is implicated as a sensitizer of peripheral nociceptive terminals during inflammation. Glutamate is synthesized from glutamine in peripheral terminals via the enzyme, phosphate-activated glutaminase (GLS). Long-term inflammation causes increased GLS production, along with other neuropeptides, in dorsal root ganglion (DRG) neuronal cell bodies. Elevated GLS is shipped to peripheral terminals causing increased glutamate synthesis and release, contributing to peripheral sensitization in the rat adjuvant induced arthritis (AIA) model. 6-diazo-5-oxo-L-norleucine (DON), a glutamine analog, irreversibly binds to GLS and interferes with glutamate synthesis. Peripheral inhibition of GLS with DON at the onset of inflammation decreases paw edema and suppresses c-fos immunoreactivity in the rat spinal cord and application of DON three days after CFA injection has a long term analgesic effect on thermal hyperalgesia and mechanical allodynia. In the current study, we applied DON at the peripheral terminal at the onset of AIA and evaluated the paw edema and nociceptive behaviors as indicators of the severity of inflammation. The GLS expression level in rat peptide-containing DRG neurons also was evaluated at 48 h of AIA. We

hypothesized that diminished glutamate release would reduce the initial peripheral sensitization of inflammation and attenuate the increase in GLS in DRG neurons during the development of inflammation. In this study, unilateral AIA was induced with a single subcutaneous injection of complete Freud's adjuvant (CFA) into the rat hindpaw. DON was injected twice intraplantar at 12 h and 30 min prior to the CFA-injection. Control rats received intraplantar vehicle injection before CFA. The sensitivity to thermal and mechanical stimuli was evaluated by paw thermal withdrawal latency (TWL) and paw pressure sensitivity test. Paw size was also evaluated as an index of the severity of inflammation. At 48 h of AIA, ipsilateral L4 DRGs were collected and colocalization of GLS with calcitonin-gene peptide (CGRP) or substance P (SP) was performed using immunofluorescence microscopy. Mean grey intensity of GLS, CGRP and SP were evaluated with image analysis. It was found that administration of DON at the site of inflammation alleviated the thermal hyperalgesia and edema and suppressed the elevation of GLS-ir in DRG neuronal cell bodies at 48 h AIA, especially in small diameter, peptide-containing neurons. Our result supports the notion that glutamate production and release at the onset of inflammation activates nociceptors and contributes to the generation of nociceptive hypersensitivity. Glutamate release also drives the alteration of neurochemical components in the DRG neuronal cell bodies that are related to nociceptive neurotransmission that leads to the development of chronic pain.

Introduction

Glutamate is the major excitatory neurotransmitter in primary sensory neurons and has received attention recently as a sensitizer for nociceptive transmission and hypersensitivity (Hammerschlag and Weinreich, 1972, Johnson, 1972, al-Ghoul et al., 1993, Miller et al., 2011). At the peripheral branch of primary afferents, endogenous glutamate is released from nerve terminals in response to natural or electrical stimuli, tissue or nerve injury, and chemical activation (Wheeler et al., 1966, Omote et al., 1998, deGroot et al., 2000, Zahn et al., 2002, Jin et

al., 2009). In various animal nociceptive models, rapid excessive glutamate production is found during the acute and chronic phases in the targeted tissue including skin, knee joint and muscle (Omote et al., 1998, Lawand et al., 2000, McNearney et al., 2000). Injection of exogenous glutamate and specific glutamate receptor agonists causes a quick-onset inflammatory pain by activation of excitatory amino acid receptors (EAAR) located on the nociceptive nerve terminal in an auto-/para-stimulation fashion (Carlton, 2001). These studies demonstrate that glutamate contributes to the generation of inflammation pain and peripheral sensitization.

Thus far, almost all ionotropic and metabotropic EAARs have been detected at the peripheral nerve terminal and it is likely that endogenous glutamate activates nociceptors via biding to glutamate receptors in an auto-/para-stimulation manner (Sato et al., 1993, Carlton et al., 1995, Coggeshall and Carlton, 1998). Glutamate acts to activate primary afferent neurons during inflammation and the use of specific or non-specific EAAR antagonists provides analgesic effects in different models of pain, including CFA-/carrageenan-/formalin/capsaicin-induced inflammation, postoperation, and nerve injury (Ren and Dubner, 1993, Lawand et al., 1997, Beirith et al., 2002, Zahn et al., 2002, Nakayama et al., 2010). The multiplicity of glutamate receptor expression levels at the peripheral nerve terminals, however, makes it hard to achieve effective pain relief with a single EAAR antagonist (Zhou et al., 1996). Moreover, among the studies on glutamate's role in nociceptive transmission, few investigations have been performed on evaluating or modifying the production of glutamate in regard to the glutamate-glutamine cycle. Comparable to the glutamatergic neurons in the CNS, the glutamate-releasing primary sensory neurons in peripheral nervous system have limited ability to *de novo* synthesis of glutamate from α-ketoglutarate from the TCA cycle (Hamberger et al., 1979a, Hamberger et al., 1979b, Peng et al., 1993, Hertz et al., 1999, Hertz, 2004, Waagepetersen et al., 2005). Primary sensory neurons use glutaminase (GLS) as the major synthetic enzyme to replenish the neurotransmitter pool for glutamate synaptic release (Kvamme et al., 2001, Waagepetersen et al.,

2005). 6-diazo-5-oxo-L-norleucine (DON) is a glutamine analog that irreversibly binds to the glutamine-binding site of glutaminase and thus blocks the conversion from glutamine to glutamate (Miller et al., 2011). Previous studies from our laboratory have shown that peripheral inhibition of GLS with DON at the onset of carrageenan-induced inflammation inhibits thermal hyperalgesia and suppresses c-fos immunoreactivity in the rat spinal cord (Hoffman and Miller, 2010), which supports the notion that peripheral glutamate release contributes to the generation of pain.

Acute release of glutamate at the site of tissue injury may mainly depend on rapid exocytosis of synaptic vesicles mediated by Ca^{2+} influx into nerve terminals in response to increased neuronal activity. Glutaminase activity can also be up-regulated by calcium entry into the nerve terminal for replenishment of synaptic vesicles. A long-term mechanism, however, that leads to increased glutamate production is expected for elevated glutamate levels in peripheral nerve fibers during chronic pain. Over-activation of primary afferents at the initial phase of inflammation drives alterations in DRG neuronal cell bodies for maintenance of pain, a cellular process referred to as a "phenotypic switch" (Woolf and Ma, 2007). Additional studies from our laboratory show that glutaminase expression and enzyme activity are elevated at DRG neuronal cell bodies and peripheral terminals during chronic adjuvant-induced arthritis (AIA) (Miller et al., 2012). GLS inhibition by peripheral application of DON, three days after CFA injection, has a long term analgesic effect on thermal hyperalgesia and mechanical allodynia (Miller et al., 2010). These studies indicate a positive regulatory mechanism between glutamate and its synthetic enzyme glutaminase contributing to the modulation and maintenance of pain during the chronic phase of inflammation. Considering the effects of glutamate on the generation, modulation and maintenance of peripheral sensitization at different time scales during inflammatory events, we propose that blocking the production of glutamate in the peripheral tissue prior to inflammation

may 1) alleviate the nociceptive hypersensitivity; 2) inhibit the inflammation-induced GLS elevation in the peptide-containing DRG neuronal cell bodies.

Materials and methods

Animals and induction of adjuvant-induced arthritis (AIA)

Male Sprague-Dawley rats (350 - 450 g) were housed in a 12:12 light/dark cycle and given free access to food and water *ad libitum*. The behavioral testing facility was maintained at room temperature of 21-22°C with humidity at 40%. All experiments were performed in a darkened room during in the light phase on independent groups of animals and a desk lamp was used to illuminate the testing area to avoid agitation from the direct light. Rats were acclimatized at least for 3 days in the behavioral testing facility and habituated to handling and the testing equipment before the experiment. Procedures were carried out in accordance with the National Institutes of Health Guide for the care and use of laboratory animals and approved by the committee of animal care and use for research at Oklahoma State University Center for Health Sciences. All efforts were made to minimize the number of animals used and their suffering.

Drugs and adjuvant-induced arthritis (AIA)

6-diazo-5-oxo-l-norleucine (DON, Sigma-Aldrich, U.S.) was dissolved in sterile 0.01 M sodium phosphate buffered saline (PBS) and injected as 20μmol/25μl or 10μmol/25μl using a 25-μl Hamilton syringe with the 32-gauge needle (Hamilton, USA). Doses of DON were chosen based on previous behavioral experiments (Miller, 2007). DON was injected twice prior to CFA-injection: 12 hours and 30 minutes prior to the CFA-injection (Hoffman and Miller, 2010). Solutions were prepared fresh before the 1st injection and kept from light at 4 °C for the 2nd injection. The same amount of sterile 0.01 M PBS was injected in rats as a vehicle control group.

Rats were anesthetized with isoflurane (initially 5%, then reduced to 2.5% following induction of anaesthesia, in 3l/min O_2) prior to all injections. All injections were given subcutaneously into the plantar hindpaw. Adjuvant-induced arthritis (AIA) was induced by a single unilateral subcutaneous injection of 75 µL of complete Freund's adjuvant (CFA; Mycobacterium butyricum, Sigma) emulsified in saline (1:1) in the hindpaw plantar surface of the rats. Naïve rats were anaesthetized with isoflurane, but not injected. A 2×3 design giving six groups (n=6 per group) was formed and listed in the table below (N=6 per group): 1) naïve, 2) naïve + vehicle, 3) naïve + DON, 4) CFA-only, 5) CFA + vehicle, and 6) CFA + DON. Rats were tested for the effect of pre- and concurrent application of 20 µmol/25µl or 10 µmol/25µl solutions to measure nociceptive behaviors at various time points after the initiation of AIA: thermal withdrawal latency, mechanical threshold and paw thickness. Rats receiving 20 µmol/25µl DON were evaluated for 8 days of AIA, whereas rats receiving 10 µmol/25µl DON were evaluated for 2 days of AIA.

Behavioral studies

The rats were tested for 3 days prior to the CFA-injection. Tests started after cessation of exploratory behavior. In both thermal and mechanical tests, the average of three measurements was considered as a mean measurement at a specific time point. A 5-minute resting period occurred between measurements.

Ipsilateral hind paw metatarsal thickness was measured using a dial caliper (Mitutoyo, Japan) as an index of the severity of inflammation. Nociceptive threshold to thermal stimuli was determined by measuring thermal withdrawal latency (TWL) using Plantar Thermal apparatus (Ugo Basile, Italy) at an intensity of 55 mW/cm^2. The TWL was recorded automatically by the instrument from the onset time of the radiant heat to the time of withdrawal of the rat hindpaw. The maximum exposure time was set at 32 seconds to prevent tissue damage. Nociceptive threshold to mechanical stimuli was determined by measuring the mechanical response using the Dynamic Plantar Aesthesiometer (Ugo Basile, Italy). The force of the metal filament increased

from 0 to 50 grams within 20 seconds. The force was recorded by the instrument when the animal moved its paw or if the maximum force (50 g) was met.

Immunohistochemistry (IHC)

An additional 12 male rats (n=3 per group) were included for the IHC study. Since no significant changes in paw thickness and behavior tests in vehicle- and DON-treated naïve rats (Fig. 1), The IHC study only focused on 1) naïve, 2) CFA-only, 3) CFA + vehicle, and 4) CFA + DON. The dose of DON used in IHC studies was 10 μmol/25μl. At 48 h after injection of CFA, rats were processed for IHC study following previously described methods (Hoffman et al., 2010). All tissue collections were performed on the same day of experiment and processed for the IHC study under the same conditions to minimize the batch-to-batch variations introduced during experiment. Rats were anesthetized with 3 ml of Avertin (2.5% of 2, 2, 2-Tribromoethanol, Sigma-Aldrich) and 0.8 ml of xylazine (1 mg/ml, AnaSed, LLOYD). The rats were perfused with 80 ml calcium free Tyrode's solution followed by 300 ml optimized fixative for GLS immunolabeling described in previous papers (Hoffman et al., 2010). Briefly, it contains 0.2% (w/v) paraformaldehyde, 70% (v/v) picric acid, 0.1M sodium phosphate buffer at pH 7.3. Ipsilateral L4 DRGs were removed and post-fixed in the same fixative for 4 h at 4°C. Tissues were transferred to 10% sucrose in PBS, pH 7.3 overnight at 4°C. Frozen sections were cut at 10 μm. Every fifth section was used to reduce the possibility of double-counting the same neuron. Dried sections were rinsed three times with PBS. Slides were blocked in 0.5% (w/v) polyvinlypyrolideone and 0.5% bovine serum albumin in PBS with 0.3% (v/v) Triton X-100 (PBS-T, Sigma). Colocalizaition of glutaminase with CGRP or SP was performed using fluorescence immunohistochemistry. The polyclonal rabbit anti-glutaminase antiserum was a generous gift from Dr. Norman Curthoys (Colorado State University). Sections were incubated in the antisera containing rabbit-anti glutaminase (1:20,000) with mouse anti-CGRP (1:8,000; Santa Cruz) or mouse anti-SP (1:2,000; R&D Systems) for four days at 4°C. After incubation in primary antisera, sections were rinsed three times in PBS and incubated in secondary antisera

containing biotinylated goat anti-rabbit IgG (1.0 μg/ml; Vector Laboratories; Burlingame, CA, USA) and Alexa Flour 555 conjugated goat anti-mouse IgG (1.67 μg/ml; Invitrogen; Carlsbad, CA, USA) diluted in PBS-T for 1 h at room temperature. Sections were rinsed twice in PBS and once in sodium carbonate buffered saline (SCBS, pH 9.6) before incubating for 1 h in 1.0 μg/ml avidin-fluorescein isothiocyanate (FITC; Vector Laboratories) diluted in SCBS. After three rinses, sections were incubated in 300 nM 4',6-diamidino-2-phenylindole (DAPI) diluted in PBS for 10 min for nuclear staining. Slides were cover-slipped with ProLong Gold Mouting Media (Invitrogen) after three more PBS rinses.

Image Analysis

Images were captured with a 40X objective on a BX51 epifluorescence microscope (Olympus; USA) using a SPOT RT740 camera (Diagnostic Instruments; USA). The micrographs were stored as 8-bit grayscale tiff format with the pixel intensity value ranging from 0-255. The exposure time and gain combination was determined empirically for each antigen in which the dimmest regions of tissue could be discerned visually for tracing, but the pixel values in brightest regions were not oversaturated. This approach allowed images to be evaluated along the linear aspect of immunofluorescence intensity. The exposure time and gain combination for a single antigen was kept the same for all tissue sections from all animals. Five non-overlapping random fields of view were captured from at least three sections of each DRG separated by 50 μm. For each field of view, three filters were used for detection of each fluorophore: FITC (green), TRITC (Alexa Fluor 555 and 568, red), and DAPI (blue). The quantitative method to measure the immunoreactivity of each antigen has been described previously (Fang et al., 2005, Hoffman et al., 2010, Hoffman et al., 2011). Neuronal profiles (NP) were counted with the following criteria: 1) the NP was through the center of the cell with a visible nucleus that was detected in the DAPI filter; 2) the NP had an intact cytoplasm that did not touch the edge of the image or the neighboring neuron. NPs were traced with a Cintiq 21UX interactive pen display (Wacom, Japan),

using the freehand selection tool in ImageJ (National Institutes of Health, USA). The cross-sectional area (μm^2) and mean gray intensity (MGI) of pixels in the cytoplasm (excluding the nucleus) was the recorded and measured as region of interest (ROI).

Threshold MGI of CGRP- and SP-labeled DRG neuronal profiles

Our laboratory has reported previously that all DRG neurons are immunoreactive for GLS using optimized fixation (Miller et al., 1993, Hoffman et al., 2011), therefore no GLS-MGI threshold was determined in the current study.

A threshold MGI for CGRP and SP was established to determine if a single NP was considered immune-positive (+ve) or –negative (-ve). The threshold MGI value of each antigen was determined from the naïve control group, since several studies reported that the proportion of neuropeptide-containing neurons can alter after peripheral inflammation (Hanesch and Schaible, 1995, Neumann et al., 1996). The detailed method was described in Chapter II. Briefly, to determine CGRP-/SP-MGI threshold, the MGI of each NP was ranked from the smallest to the largest (Chapter II, Figure 1A). The rank and MGI were plotted in x- and y-axis respectively. Based on the ranking, a CGRP-MGI threshold was set at 10 and SP-MGI threshold was set at 25. Using this method, the proportion of CGRP+ve and SP+ve NPs was 36.1% and 26.3% respectively, which is in agreement with previous studies (Lawson, 1995, Lawson et al., 1997, Lawson et al., 2002). In the following analyses, the NPs were divided by their cross-sectional area: small- (<400 μm^2; diameter, <23 μm) and medium- (400-800 μm^2; diameter, 23-32 μm) and large-sized (>800 μm^2; diameter, >32μm) (Fang et al., 2005, Hoffman et al., 2010).

Statistical Analysis

Data from the image analyses were reported as mean ± standard error of the mean. All data were subjected to the Kolmogorov-Smirnov test to determine the normality of the distribution. All data from behavioral tests passed the normality test and were analyzed with two-way ANOVA by ranks followed by Bonferroni post-tests. The MGI values from the IHC study were not normally distributed and the variances were heterogenous for nearly all comparison. Nonparametric

statistical analyses, therefore, were applied and the median is shown in the dot plots. Significant differences in MGI among different experimental conditions were determined by the Kruskal-Wallis one-way ANOVA by ranks followed by *Dunn posthoc* test (Prism version 5.03, GraphPad Software Inc., USA). In all analyses, $P < 0.05$ were considered significant.

Results

Behavioral studies

To study the effect of glutamate synthesis inhibition on nociceptive behaviors in response to inflammation, behavioral studies were performed to measure the paw thickness, thermal withdrawal latency to noxious heat stimulus, and mechanical threshold to paw pressure stimulus. Studies presented in Fig. 1 used a dose of DON at 20 μmol/25μl. Metatarsal paw thickness was measured as an indicator of the severity of the inflammation. The baseline thickness of the paw ranged from 5.1 to 5.5 mm and no statistical difference was detected among the baseline measurements of each experimental group (Fig. 1A). Within 6 hours following intraplantar CFA-injection, swelling of the hindpaw was observed in all three CFA-treated groups (CFA-only, CFA+vehicle, and CFA+DON). The hindpaw thickness of CFA-only and CFA+vehicle groups was significantly higher than the baseline measurement and those of the non-inflamed groups (Fig. 1A). It reached a maximal thickness at 24 h post-inoculation and remained elevated during the 8-days of the current study. Injection of vehicle or DON did not change the paw thickness of the non-inflamed groups. Pretreatment with DON, but not saline vehicle, significantly attenuated the swelling in inflamed rat hindpaw compared to CFA-only group at 6 h (19.0%, $p < 0.001$), 12 h (15.5%, $p < 0.001$), 24 h (10.0%, $p < 0.05$) and 48 h (17.8%, $p < 0.05$), but not at day 4 or day 8 AIA (Fig. 1A).

Injection of CFA produced a significant decrease in TWL to radiant thermal stimulus within 3 h post-CFA injection. The decrease in TWL was interpreted as the presence of thermal hyperalgesia. Injection of saline vehicle or DON in naïve rats did not produce thermal hyperalgesia.

Pretreatment with DON, but not the saline vehicle significantly attenuated thermal hyperalgesia compared to CFA-only group at 48 h (19.0%, $p < 0.05$), 4 d (19.0%, $p < 0.05$), and 8 d (19.0%, $p < 0.05$) AIA (Fig. 1B). The TWL approached the naïve control level at these time points, indicating a long-term analgesic effect of DON on thermal hyperalgesia.

Injection of CFA produced a significant decrease in paw pressure threshold to mechanical stimulus within 3 h post-CFA injection. The decrease in paw pressure threshold to mechanical stimulus was interpreted as mechanical allodynia. Injection of saline vehicle or DON in naïve rats did not produce mechanical allodynia. No statistical difference was detected at any time point in mechanical allodynia, but a trend toward alleviation in mechanical allodynia was observed in the CFA+DON group when compared to CFA-only group (Fig. 1B).

Based on the studies from 0 to 8 days AIA, 48 h of AIA was chosen to study the effect of DON on protein expression in the DRG neuronal cell bodies. A lower dose of DON was used in this study (10 μmol/25μl, Fig. 2). Pre-treatment with 10 μmol/25μl of DON achieved inhibitory effects on paw edema from 6 h to 48 h AIA (Fig. 2A) and thermal hyperalgesia (Fig. 2B) was significantly reversed at 48 h similar to the higher dose of DON (20 μmol/25μl, Fig. 1B and 2B). No differences were observed in mechanical allodynia with CFA + DON treatment at 10 μmol/25μl.

Qualitative results of temporal alteration of SP-, CGRP-, and GLS-ir during AIA

To study the effect of peripheral inhibition on glutamate on neuropeptide and glutaminase expression in the DRG neuronal cell bodies, IHC was performed to evaluate the immunoreactivity of those proteins in subpopulations of DRG somata. Figure 3 shows representative photomicrographs from the IHC studies. In the naïve control group, all DRG neurons expressed GLS (Fig. 3A-3H). CGRP (Fig. 3A'-3D') and SP (Fig. 3E'-3H') were mainly expressed in neurons with small to medium sized neuronal cell bodies and these neurons were co-labeled with GLS (indicated with arrowheads in Fig. 3A"-3H").

Neuropeptides are expressed in subpopulations of DRG neurons, so a MGI threshold was set to determine the positive (+ve) and negative (-ve) as described in the Methods. In naïve control rats, the basal proportion of CGRP- and SP-expressing neurons is 36.1% and 26.3% respectively (Fig. 4). An increased proportion of CGRP-labeled NPs was observed at 48 h after the onset of AIA ($p < 0.01$), while peripheral DON injection completely inhibited the proportional increase produced by AIA (Fig. 4). Alteration of CGRP-MGI was not detected at 48 h of AIA, however, CGRP-MGI in CFA + DON group was significantly decreased (13.8%, $p<0.01$) compare to basal CGRP-MGI in the naïve group (Fig. 5). Further dividing the NPs by their cross-sectional area, It was found that a decrease in the proportion of medium-sized neurons and MGI of small-sized CGRP-labeled NPs from rats with AIA treated with DON compared to AIA rats (Fig. 5). No change in proportion or MGI was detected in SP-labeled DRG NPs at 48 h of AIA (Fig. 4,5).

AIA significantly elevated the GLS-MGI in all DRG NPs compared to naïve animals (29.2%, $P<0.001$) and the increased GLS-MGI was significantly suppressed by peripheral DON injection (Fig. 6). When further dividing the NPs by their cross-sectional area, It was found that that the inhibitory effect of DON was observed in all three-sized groups (Fig. 6).

Discussion

By measuring the plantar thickness and nociceptive behavioral parameters, the present study confirmed that unilateral intraplantar CFA-injection produced rapid and persistent local inflammatory responses, including paw edema, thermal hyperalgesia, and mechanical allodynia. The peripheral inhibition of glutamate production at the onset of inflammation indicates that: 1) it attenuates the edema formation and has an anti-edemic effect lasting at least 48 h after CFA injection; 2) it reverses the thermal hyperalgesia to baseline level from 48 h to 8 days AIA; 3) it shows a trend toward alleviation of mechanical allodynia. Additional studies using immunofluorescent microscopy and image analysis demonstrated the effect of peripheral glutamate release during inflammation on the phenotypic plasticity in the DRG neuronal cell bodies. For example, these results showed that elevated immunoreactivity of glutaminase,

calcitonin gene-related peptide (CGRP), and substance P (SP) were decreased during inflammation with peripheral application of DON. These results indicate that peripheral release of glutamate contributes to the modulation and maintenance of pain and serves as one of the mechanisms underlying the "phenotypic switch" for the development of chronic pain.

Source of peripheral glutamate during the initial phase of inflammation

Besides the role as the major excitatory neurotransmitter in the nervous system, glutamate is a non-essential amino acid serving as a key component in cellular biosynthesis and energy metabolism, which suggests that glutamate exists in both neuronal and non-neuronal tissues (Skerry and Genever, 2001). It raises the possibility that the non-neuronal cell types around the inflammation site may contribute to the elevated glutamate concentration. Glutamate is produced in many non-neuronal cell types carrying metabolic or signaling functions, e.g. bone, keratinocyte, neutrophils, macrophages, and mast cells (Lawand et al., 2000, Skerry and Genever, 2001). Whether these cells produce glutamate via glutaminase or *de novo* synthesis of glutamate from α-ketoglutarate from the TCA cycle has not been examined thoroughly and thus our studies cannot exclude these local or infiltrating cells as the sources of peripheral glutamate (Hamberger et al., 1979a, Hamberger et al., 1979b, Peng et al., 1993, Hertz et al., 1999, Hertz, 2004, Waagepetersen et al., 2005). One of the major goals in our studies is the pharmacological intervention targeting the glutamate production and the effect on edema and hyperalgesia therefore the source of glutamate in the peripheral tissue is not examined in the current study. Several studies, however, support the idea that the major source of peripheral glutamate is from peripheral nerve terminals. In the current study, unilateral intraplantar CFA-injection produces a localized inflammation that develops over minutes and lasts for more than a week (Ren and Dubner, 1999). Several lines of studies support that, during the initial phase of AIA, the source of glutamate in the inflamed tissue is the primary afferents. Firstly, pre-administration of lidocaine, blocking all neuronal activity via fast voltage-gated sodium channels, completely prevents the

increase in extracellular glutamate concentration in a kaolin/carrageenan-induced inflammation model for at least 4 hours (Lawand et al., 2000). This indicates that the increased glutamate concentration is dependent on peripheral neuronal discharge produced by inflammation. Secondly, in the nerve terminal, vesicular glutamate transporter (VGLUT) is the crucial protein that packages glutamate into synaptic vesicles. Glutamate release occurs from synaptic vesicles via a Ca^{2+}-mediated exocytotic mechanism, but not from the cytoplasmic pool. Pre-administration of Chicago sky blue B6, a VGLUT blocker, attenuates the glutamate-evoked paw edema (Beirith et al., 2002). This indicates that exogenous glutamate released during glutamate-evoked inflammation is mediated via VGLUT's and that glutamate contributes to the edema in neurogenic inflammation. Thirdly, 6-diazo-5-oxo-L-norleucine (DON) specifically targets glutaminase (phosphate-activated glutaminase, PAG). The inhibitory effects of DON on edema (Fig. 1A and 2A) and nociceptive behaviors (Fig. 1B, 1C, 2B, and 2C) in carrageenan-and CFA-induced inflammation have been achieved by the reducing glutamate production via inactivation of glutaminase (Hoffman and Miller, 2010, Miller et al., 2010). It is reasonable, therefore, to assume that the primary sensory nerve terminal is a major source of elevated glutamate during the initial phase of acute inflammation.

AIA is produced by injection of an inactive *Mycobacterium tuberculosis* suspension into healthy tissue to mimic an arthritic-like inflammation. The heat-killed bacteria may activate the innate immune system and exacerbate the inflammation via a non-neurogenic mechanism (Billiau and Matthys, 2001, Chiu et al., 2012). While proceeding to the chronic phase, infiltrating leukocytes and other necrotic cells may release free glutamate around primary nerve terminals, making the extracellular neurochemical environment more complex. Chronic inflammation also produces elevation of glutaminase and glutamate in sensory nerve fibers of the skin. Peripheral injection of DON at 3 day AIA has a robust inhibitory effect on thermal hyperalgesia and mechanical allodynia (Miller et al., 2010) and significantly reduces glutamate immunoreactivity in peripheral

nerve terminals. These results support that the elevated glutaminase at the peripheral nerve terminal is a major source of glutamate during the chronic phase of inflammation.

Possible role of glutamate on edema during acute inflammation

Primary afferent nociceptors carry "efferent" functions during inflammation. At the peripheral branch of primary afferents, inflammatory mediators including glutamate, CGRP and SP are released from nerve terminals via synaptic vesicles in response to natural or electrical stimuli, tissue or nerve injury, and chemical activation (Donnerer et al., 1992, Maggi, 1995, Juranek and Lembeck, 1997). CGRP and SP are two neuropeptide markers for "peptide-containing" nociceptive neurons (Woolf and Ma, 2007). The peripheral effects of the neuropeptides include SP's effect on plasma exudation and CGRP action on arterioles causing vasodilation and increased local blood flow (Holzer, 1998). At the peripheral terminal, these peptides are considered as the major initiators and contributors to neurogenic inflammation (Moussaoui et al., 1993, Holzer, 1998). At peripheral terminals, there also is interaction of these ligands with their receptors. Unmyelinated C-fiber nerve terminals express SP receptors and ionotropic glutamate receptors (NMDAR, AMPAR and KAR) (Beirith et al., 2002, Ferreira et al., 2005). Co-injection of SP with glutamate in the hind paw significantly potentiates the duration of nociceptive behavioral responses compared to that seen following injection of either substance alone (Carlton et al., 1998). This suggests that SP can enhance glutamate-induced nociceptive behaviors. CGRP is not directly involved in the paw edema or nociception by glutamate injection, but, instead, CGRP contributes to neurogenic inflammation by causing vasodilation and facilitating SP produced vascular permeability (Beirith et al., 2002). In the current study, the anti-edemic effect of glutamate synthesis inhibition suggests a rapid release of glutamate occurring at the time of the CFA-injection (Fig 1A and 2A). At the central terminal in the spinal cord dorsal horn, SP and

CGRP are co-released with glutamate, augment glutamate efflux, enhance glutamatergic synaptic strength and, as a result, increase glutamate signaling in the nociceptive transmission pathway (Otsuka and Konishi, 1976, De Biasi and Rustioni, 1988, Kangrga et al., 1990, Kangrga and Randic, 1990, Okano et al., 1998). The assumption that SP and CGRP are co-packaged with glutamate in the large dense-core vesicle (LDCV) is yet to be confirmed at the peripheral terminal. Alternatively, glutamate may be synaptically released alone via small, clear vesicles causing activation of the nerve terminal by binding to EAA-Rs, and triggering the exocytosis of peptide-containing LDCVs.

From 48 h to 8 days AIA, the anti-edemic effect of DON became less obvious (Fig. 1A and 2A). A first explanation is that the initial tissue insult promotes the protein synthesis of the neuropeptides in the DRG neuronal cell bodies and the newly synthesized proteins arrive at peripheral nerve terminal after anterograde transport to contribute to the maintenance of edema during the chronic phase of AIA. A second explanation is that after 48 h, the inflammatory mediators released during the initial neurogenic phase cause leukocyte infiltration, which in turn add a non-neurogenic component at the inflamed site by secreting interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and nerve growth factor (NGF) (Chiu et al., 2012). Release of pro-inflammatory mediators around primary nerve terminals may intensify the inflammatory response and activate the nerve terminals independent of glutamate signaling pathways.

Possible role of glutamate on nociceptive behaviors during acute inflammation

Pain can be classified as acute and chronic pain. Some acute pain is followed by chronic pain and chronic pain usually is preceded by acute pain. As in other pathological diseases, the development of pain involves generation, modulation, and maintenance. Even though most of these events are temporally overlapping, many studies have been devoted to identifying the key factors and mechanisms responsible for exacerbating the initial painful stage at different pain phases. With

the progressive understanding of the mechanisms in pain, it seems that most of the factors and their contributions are highly integrated and overlapping. The following section identifies some of the most prevalent perspectives in the field to stress the role of glutamate in the generation, modulation and maintenance of pain.

Various biological and pharmacological studies demonstrate that all EAARs including ionotropic (iGluR) and metabotropic (mGluR) EAARS are expressed at peripheral nerve terminals (Sato et al., 1993, Miller et al., 2011). EAARs co-localize with Aδ- and C-fiber nerve terminals containing transient receptor potential cation channels, subfamily vanilloid member1 (TRPV1) (Medvedeva et al., 2008, Jin et al., 2009). TRPV1 is the key transducer protein that responds to noxious thermal stimuli, the exogenous ligand capsaicin, protons, and other endogenous ligands (Basbaum et al., 2009). TRPV1 channel function in peripheral terminals is dependent on the glutamatergic signaling pathways. For example, deletion of VGLUT2, the key protein for glutamate synaptic release on capsaicin-sensitive nerve terminals, leads to impaired capsaicin-induced thermal hyperalgesia (Lagerstrom et al., 2010). Moreover, peripheral administration of NMDA and non-NMDA iGluR antagonists significantly attenuate thermal hyperalgesia in capsaicin-/carrageenan-/formalin-/CFA-induced inflammation (Ren and Dubner, 1993, Leem et al., 2001, Jin et al., 2009). One mechanism underlying thermal hyperalgesia is that the initial activation of TRPV1 by noxious heat/endogenous ligands allows for calcium [Ca^{2+}] influx to initiate the transducer potential. The transducer potential summates and produces a generator potential via the voltage-gated sodium channels (Nav1.7, Nav1.8, and Nav1.9). The generator potential depolarizes the nerve terminal and triggers the release of glutamate. Glutamate then activates the iGluRs and Group II/III mGluRs at the nociceptive nerve terminals in an autocrine/paracrine manner (Medvedeva et al., 2008, Carlton et al., 2009, Shoudai et al., 2010, Carlton et al., 2011). Activated iGluRs result in strengthening the effects of noxious signals by decreasing the neuronal discharge threshold and increasing responsiveness to noxious stimuli, one

form of sensitization described as primary “thermal hyperalgesia.” In the current study, the effect of DON most likely diminishes available glutamate at the peripheral terminals and un-couples the increase in neuronal activity exerted by glutamate receptors on primary afferents (Fig. 1B and 2B). One noticeable item is that the inhibitory effect of DON, regardless of high or lose dose, on thermal hyperalgesia occurs at 48 h after the onset of AIA (Fig. 1B and 2B). This is a delayed effect compared to the anti-edema effect that occurs as early as 6 h AIA (Fig. 1A and 2A). The exact mechanism of the delayed inhibitory effect on thermal hyperalgesia is not clear, but several assumptions are proposed for future studies. First, the pharmacological properties of DON has not been fully characterized and the 48 h delay may indicate the time for uptake by the nerve terminal, the exchange into the mitochondria membrane, and eventual inhibition on mitochondrial glutaminase. Second, the thermal TWL in CFA + DON group using both concentrations appeared longer than the CFA-only and CFA + vehicle groups (Fig. 1B and 2B, 6 h – 48 h), but reached statistical significance only at 48 h. It is possible an increase in sample size might reveal inhibitory effects at earlier time points.

The transducer protein for noxious mechanosensation has not been identified definitively. The peripheral mechanism of mechanical allodynia observed in inflammation might involve a 'mechanochemical' process whereby stretch evokes the release of adenosine 5'-triphosphate (ATP) binding to P2X receptors from the damaged tissue that then excites nearby primary sensory nerve terminals (Tsuda et al., 2000). Peripheral administration of various receptor antagonists show minor effects on hypersensitivity to mechanical stimuli compared to that of central administration leading to the prevalent view in the field that mechanical allodynia is primarily a central mechanism, termed “central sensitization” (Fagan and Cahusac, 2001, Leem et al., 2001, Hama et al., 2003, Puttfarcken et al., 2010, Inquimbert et al., 2012). Studies demonstrate that central spinal glutamate receptors contribute to the increased sensitivity to mechanical stimuli. Peripheral inflammation causes increased neuronal discharge of nociceptors

and triggers the release of glutamate from presynaptic nociceptive nerve terminals in the spinal cord dorsal horn (Puttfarcken et al., 2010, Shoudai et al., 2010, Inquimbert et al., 2012). Studies using the formalin-induced inflammation model show that peripheral inflammation causes a rapid onset of amino acid release in the spinal cord dorsal horn within 10 min following injection and a late onset of glutamate release from 8-10 h following injection (Sluka and Westlund, 1993, Crown et al., 2006). Peripheral inflammation also activates expression of c-fos, an immediate early gene product and marker of activated neurons, in spinal cord dorsal horn neurons involved in nociceptive pathways. Peripheral DON administration inhibits the c-fos activation in the spinal cord indicating that peripheral glutamate release activates second order neurons (Hoffman and Miller, 2010). Activation of postsynaptic dorsal horn neurons triggers NMDARs- and PKA-dependent increase in NMDAR subunit synaptic expression on the postsynaptic membrane leading to hyperfunction of spinal NMDARs and increased glutamatergic transmission in the nociceptive pathways following peripheral inflammation (Yang et al., 2009). Increased activity in nociceptors also co-releases neuropeptides with glutamate at central terminals and CGRP and SP strengthen glutamatergic synaptic plasticity in spinal cord dorsal horn neurons (Biella et al., 1991, Sluka et al., 1992). These events occurring in the central nervous system are termed "central sensitization." In the current study, we failed to detect the inhibitory effect of DON pre-treatment on mechanical allodynia, but a trend toward alleviation was observed (Fig. 1C and 2C). These results support the notion that mechanical allodynia involves more alterations at the central terminal than that of the periphery (Hama et al., 2003, Woolf and Ma, 2007). Alternatively, it is possible that the sample size of the current studies (n=6 for 20 μmol/25μl in Fig. 1C, and n=3 for 10 μmol/25μl in Fig. 2C) is not sufficient to achieve the statistic significance for detecting changes in mechanical sensitivity.

In summary, pre-inhibition of glutamate production achieved a moderate anti-edemic effect and inhibitory effect on thermal hyperalgesia during AIA. These results indicate that glutamate

release occurs from peripheral nerve terminals after induction of inflammation prior to the exhibition of the cardinal inflammatory symptoms. It suggests that glutamate has an important role in facilitating the generation and development of neurogenic inflammation and nociceptive responses.

Possible role of peripheral glutamate on phenotypic switch and persistent pain during chronic inflammation

As mentioned in the previous section, injection of CFA initiates a rapid-onset neurogenic inflammatory response characterized by release of glutamate, neuropeptides, and other inflammatory mediators from the primary afferent nerve terminals. As a result, inflammatory mediators sensitize peripheral nerve ending to decrease the threshold and increase responsiveness to sensory stimulation. These events generate noxious retrograde signals in peripheral nerve axons including increased and altered action potentials and retrograde transport of neurotrophic factors to "notify" the neuronal cell bodies in the DRG of inflammatory events in the periphery (Woolf and Ma, 2007). Retrograde signals drive the alteration of neurochemical components in the soma of nociceptive neurons by changing the transcription, translation, trafficking of proteins. In DRG neurons, the major protein synthesis occurs in the neuronal cell bodies and the newly synthesized proteins are delivered to the nerve terminal via anterograde transport. Our previous studies have demonstrated that glutaminase expression in the neuronal cell bodies is significantly elevated at 1 and 2 days of AIA followed by a gradual decrease from 4 day to 8 day of AIA (See Chapter II) suggesting an anterograde transport to the periphery (See Chapter III) and/or spinal cord. Increased GLS in the peripheral terminals during the chronic phase of AIA indicates that a prolonged increase in glutamate production occurs in the terminals. This "positive feedback" mechanism may be one of the mechanisms that explain the chronic painful sensations and elevated glutamate concentrations found in the knee joint in several chronic arthritic pain models (Lawand et al., 2000, McNearney et al., 2000, McNearney et al., 2004). Studies on EAARs at 48

h AIA showed an increase in EAAR immunolabeling in lightly myelinated Aδ- and unmyelinated C-fiber nerve fibers, suggesting an increased capability in glutamate signaling pathways in peripheral nociceptors (Carlton and Coggeshall, 1999). When pre-depleting glutamate at peripheral nerve terminals with DON, decreased glutaminase expression was observed in DRG neurons across small-, medium-, and large-sized categories at 48 h AIA (Fig. 5A-5H). The attenuation of increased GLS in the neuronal cell body during AIA would result in decreased GLS axonal transport with decreased glutamate synthesis and release at the peripheral terminal. By attenuating increases in availability of the GLS, glutamate's synthetic enzyme, the endogenous ligand of EAARs is significantly reduced at the chronic phase of AIA. This may be one of the mechanisms underlying the long-term analgesic effect of DON.

Conclusions

By peripheral inhibition of glutamate production and release with DON at the onset of inflammation, these results confirm other studies indicating that glutamate contributes to the generation of local neurogenic inflammation and peripheral sensitization. Peripheral application of DON also inhibits the elevated glutaminase in DRG neuronal cell bodies induced by peripheral inflammation. This indicates that, during inflammation, glutaminase is axonally transported to the peripheral nerve terminal to enhance glutamate production and release for the maintenance of nociceptor sensitivity. This is may be one mechanism underlying the long-lasting analgesia effect of DON on the nociceptive behaviors during the chronic phase of pain in the AIA model (Miller, 2007). These observations indicate that peripheral glutamate production and release could be altered by pharmacological intervention of glutaminase enzyme activity in the nerve terminal, a new strategy for manipulation for glutamate-mediated nociceptive transmission.

References

al-Ghoul WM, Volsi GL, Weinberg RJ, Rustioni A (1993) Glutamate immunocytochemistry in the dorsal horn after injury or stimulation of the sciatic nerve of rats. Brain research bulletin 30:453-459.
Basbaum AI, Bautista DM, Scherrer G, Julius D (2009) Cellular and molecular mechanisms of pain. Cell 139:267-284.
Beirith A, Santos AR, Calixto JB (2002) Mechanisms underlying the nociception and paw oedema caused by injection of glutamate into the mouse paw. Brain research 924:219-228.
Biella G, Panara C, Pecile A, Sotgiu ML (1991) Facilitatory role of calcitonin gene-related peptide (CGRP) on excitation induced by substance P (SP) and noxious stimuli in rat spinal dorsal horn neurons. An iontophoretic study in vivo. Brain research 559:352-356.
Billiau A, Matthys P (2001) Modes of action of Freund's adjuvants in experimental models of autoimmune diseases. Journal of leukocyte biology 70:849-860.
Carlton SM (2001) Peripheral excitatory amino acids. Current opinion in pharmacology 1:52-56.
Carlton SM, Coggeshall RE (1999) Inflammation-induced changes in peripheral glutamate receptor populations. Brain research 820:63-70.
Carlton SM, Du J, Zhou S (2009) Group II metabotropic glutamate receptor activation on peripheral nociceptors modulates TRPV1 function. Brain research 1248:86-95.
Carlton SM, Hargett GL, Coggeshall RE (1995) Localization and activation of glutamate receptors in unmyelinated axons of rat glabrous skin. Neuroscience letters 197:25-28.
Carlton SM, Zhou S, Coggeshall RE (1998) Evidence for the interaction of glutamate and NK1 receptors in the periphery. Brain research 790:160-169.
Carlton SM, Zhou S, Govea R, Du J (2011) Group II/III metabotropic glutamate receptors exert endogenous activity-dependent modulation of TRPV1 receptors on peripheral nociceptors. The Journal of neuroscience : the official journal of the Society for Neuroscience 31:12727-12737.
Chiu IM, von Hehn CA, Woolf CJ (2012) Neurogenic inflammation and the peripheral nervous system in host defense and immunopathology. Nature neuroscience 15:1063-1067.
Coggeshall RE, Carlton SM (1998) Ultrastructural analysis of NMDA, AMPA, and kainate receptors on unmyelinated and myelinated axons in the periphery. The Journal of comparative neurology 391:78-86.
Crown ED, Ye Z, Johnson KM, Xu GY, McAdoo DJ, Hulsebosch CE (2006) Increases in the activated forms of ERK 1/2, p38 MAPK, and CREB are correlated with the expression of at-level mechanical allodynia following spinal cord injury. Experimental neurology 199:397-407.
De Biasi S, Rustioni A (1988) Glutamate and substance P coexist in primary afferent terminals in the superficial laminae of spinal cord. Proc Natl Acad Sci U S A 85:7820-7824.
deGroot J, Zhou S, Carlton SM (2000) Peripheral glutamate release in the hindpaw following low and high intensity sciatic stimulation. Neuroreport 11:497-502.
Donnerer J, Schuligoi R, Stein C (1992) Increased content and transport of substance P and calcitonin gene-related peptide in sensory nerves innervating inflamed tissue: evidence for a regulatory function of nerve growth factor in vivo. Neuroscience 49:693-698.
Fagan BM, Cahusac PM (2001) Evidence for glutamate receptor mediated transmission at mechanoreceptors in the skin. Neuroreport 12:341-347.
Fang X, Djouhri L, McMullan S, Berry C, Okuse K, Waxman SG, Lawson SN (2005) trkA is expressed in nociceptive neurons and influences electrophysiological properties via Nav1.8 expression in rapidly conducting nociceptors. The Journal of neuroscience : the official journal of the Society for Neuroscience 25:4868-4878.
Ferreira J, Triches KM, Medeiros R, Calixto JB (2005) Mechanisms involved in the nociception produced by peripheral protein kinase c activation in mice. Pain 117:171-181.

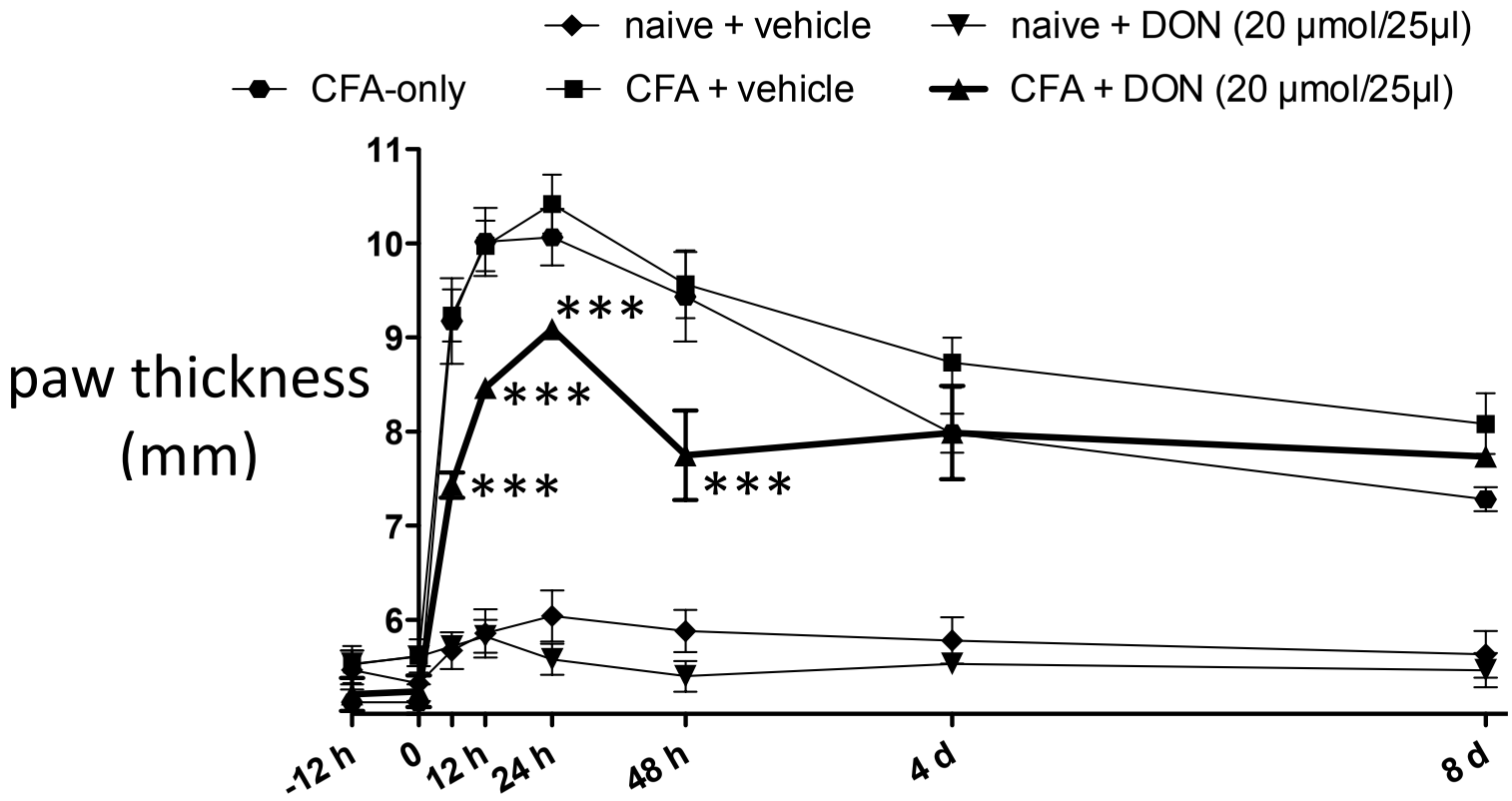
Hama A, Woon Lee J, Sagen J (2003) Differential efficacy of intrathecal NMDA receptor antagonists on inflammatory mechanical and thermal hyperalgesia in rats. European journal of pharmacology 459:49-58.
Hammerschlag R, Weinreich D (1972) Glutamic acid and primary afferent transmission. Advances in biochemical psychopharmacology 6:165-180.
Hanesch U, Schaible HG (1995) Effects of ankle joint inflammation on the proportion of calcitonin gene-related peptide (CGRP)-immunopositive perikarya in dorsal root ganglia. Progress in brain research 104:339-347.
Hoffman EM, Miller KE (2010) Peripheral inhibition of glutaminase reduces carrageenan-induced Fos expression in the superficial dorsal horn of the rat. Neuroscience letters 472:157-160.
Hoffman EM, Schechter R, Miller KE (2010) Fixative composition alters distributions of immunoreactivity for glutaminase and two markers of nociceptive neurons, Nav1.8 and TRPV1, in the rat dorsal root ganglion. J Histochem Cytochem 58:329-344.
Hoffman EM, Zhang Z, Anderson MB, Schechter R, Miller KE (2011) Potential mechanisms for hypoalgesia induced by anti-nerve growth factor immunoglobulin are identified using autoimmune nerve growth factor deprivation. Neuroscience 193:452-465.
Holzer P (1998) Neurogenic vasodilatation and plasma leakage in the skin. General pharmacology 30:5-11.
Inquimbert P, Bartels K, Babaniyi OB, Barrett LB, Tegeder I, Scholz J (2012) Peripheral nerve injury produces a sustained shift in the balance between glutamate release and uptake in the dorsal horn of the spinal cord. Pain 153:2422-2431.
Jin YH, Yamaki F, Takemura M, Koike Y, Furuyama A, Yonehara N (2009) Capsaicin-induced glutamate release is implicated in nociceptive processing through activation of ionotropic glutamate receptors and group I metabotropic glutamate receptor in primary afferent fibers. Journal of pharmacological sciences 109:233-241.
Johnson JL (1972) Glutamic acid as a synaptic transmitter in the nervous system. A review. Brain research 37:1-19.
Juranek I, Lembeck F (1997) Afferent C-fibres release substance P and glutamate. Canadian journal of physiology and pharmacology 75:661-664.
Kangrga I, Larew JS, Randic M (1990) The effects of substance P and calcitonin gene-related peptide on the efflux of endogenous glutamate and aspartate from the rat spinal dorsal horn in vitro. Neuroscience letters 108:155-160.
Kangrga I, Randic M (1990) Tachykinins and calcitonin gene-related peptide enhance release of endogenous glutamate and aspartate from the rat spinal dorsal horn slice. The Journal of neuroscience : the official journal of the Society for Neuroscience 10:2026-2038.
Kvamme E, Torgner IA, Roberg B (2001) Kinetics and localization of brain phosphate activated glutaminase. Journal of neuroscience research 66:951-958.
Lagerstrom MC, Rogoz K, Abrahamsen B, Persson E, Reinius B, Nordenankar K, Olund C, Smith C, Mendez JA, Chen ZF, Wood JN, Wallen-Mackenzie A, Kullander K (2010) VGLUT2-dependent sensory neurons in the TRPV1 population regulate pain and itch. Neuron 68:529-542.
Lawand NB, McNearney T, Westlund KN (2000) Amino acid release into the knee joint: key role in nociception and inflammation. Pain 86:69-74.
Lawand NB, Willis WD, Westlund KN (1997) Excitatory amino acid receptor involvement in peripheral nociceptive transmission in rats. European journal of pharmacology 324:169-177.
Lawson SN (1995) Neuropeptides in morphologically and functionally identified primary afferent neurons in dorsal root ganglia: substance P, CGRP and somatostatin. Progress in brain research 104:161-173.

Lawson SN, Crepps B, Perl ER (2002) Calcitonin gene-related peptide immunoreactivity and afferent receptive properties of dorsal root ganglion neurones in guinea-pigs. The Journal of physiology 540:989-1002.
Lawson SN, Crepps BA, Perl ER (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.
Leem JW, Hwang JH, Hwang SJ, Park H, Kim MK, Choi Y (2001) The role of peripheral N-methyl-D-aspartate receptors in Freund's complete adjuvant induced mechanical hyperalgesia in rats. Neuroscience letters 297:155-158.
Maggi CA (1995) Tachykinins and calcitonin gene-related peptide (CGRP) as co-transmitters released from peripheral endings of sensory nerves. Progress in neurobiology 45:1-98.
McNearney T, Baethge BA, Cao S, Alam R, Lisse JR, Westlund KN (2004) Excitatory amino acids, TNF-alpha, and chemokine levels in synovial fluids of patients with active arthropathies. Clinical and experimental immunology 137:621-627.
McNearney T, Speegle D, Lawand N, Lisse J, Westlund KN (2000) Excitatory amino acid profiles of synovial fluid from patients with arthritis. The Journal of rheumatology 27:739-745.
Medvedeva YV, Kim MS, Usachev YM (2008) Mechanisms of prolonged presynaptic Ca2+ signaling and glutamate release induced by TRPV1 activation in rat sensory neurons. The Journal of neuroscience : the official journal of the Society for Neuroscience 28:5295-5311.
Miller KE, Balbas JC, Benton RL, Lam TS, Edwards KM, Kriebel RM, Schechter R (2012) Glutaminase immunoreactivity and enzyme activity is increased in the rat dorsal root ganglion following peripheral inflammation. Pain Res Treat 2012:414697.
Miller KE, Douglas VD, Kaneko T (1993) Glutaminase immunoreactive neurons in the rat dorsal root ganglion contain calcitonin gene-related peptide (CGRP). Neuroscience letters 160:113-116.
Miller KE, Herzog B, Sutharshan M (2010) 6-Diazo-5-Oxo-L-Norleucine: A Glutaminase Inhibitor with Local Analgesic Properties. In: IASP, vol. 4193 Montreal, Qeubec, Canada.
Miller KE, Hoffman EM, Sutharshan M, Schechter R (2011) Glutamate pharmacology and metabolism in peripheral primary afferents: physiological and pathophysiological mechanisms. Pharmacol Ther 130:283-309.
Moussaoui S, Carruette A, Garret C (1993) Further evidence that substance P is a mediator of both neurogenic inflammation and pain: two phenomena inhibited either by postsynaptic blockade of NK1 receptors or by presynaptic action of opioid receptor agonists. Regul Pept 46:424-425.
Nakayama T, Naono R, Ikeda T, Nishimori T (2010) NMDA and AMPA receptors contribute to the maintenance of substance P-induced thermal hyperalgesia. Neurosci Res 67:18-24.
Neumann S, Doubell TP, Leslie T, Woolf CJ (1996) Inflammatory pain hypersensitivity mediated by phenotypic switch in myelinated primary sensory neurons. Nature 384:360-364.
Okano K, Kuraishi Y, Satoh M (1998) Involvement of spinal substance P and excitatory amino acids in inflammatory hyperalgesia in rats. Japanese journal of pharmacology 76:15-22.
Omote K, Kawamata T, Kawamata M, Namiki A (1998) Formalin-induced release of excitatory amino acids in the skin of the rat hindpaw. Brain research 787:161-164.
Otsuka M, Konishi S (1976) Substance P and excitatory transmitter of primary sensory neurons. Cold Spring Harbor symposia on quantitative biology 40:135-143.
Puttfarcken PS, Han P, Joshi SK, Neelands TR, Gauvin DM, Baker SJ, Lewis LG, Bianchi BR, Mikusa JP, Koenig JR, Perner RJ, Kort ME, Honore P, Faltynek CR, Kym PR, Reilly RM (2010) A-995662 [(R)-8-(4-methyl-5-(4-(trifluoromethyl)phenyl)oxazol-2-ylamino)-1,2,3,4-tetrahydr onaphthalen-2-ol], a novel, selective TRPV1 receptor antagonist,

reduces spinal release of glutamate and CGRP in a rat knee joint pain model. Pain 150:319-326.
Ren K, Dubner R (1993) NMDA receptor antagonists attenuate mechanical hyperalgesia in rats with unilateral inflammation of the hindpaw. Neuroscience letters 163:22-26.
Ren K, Dubner R (1999) Inflammatory Models of Pain and Hyperalgesia. ILAR journal / National Research Council, Institute of Laboratory Animal Resources 40:111-118.
Sato K, Kiyama H, Park HT, Tohyama M (1993) AMPA, KA and NMDA receptors are expressed in the rat DRG neurones. Neuroreport 4:1263-1265.
Shoudai K, Peters JH, McDougall SJ, Fawley JA, Andresen MC (2010) Thermally active TRPV1 tonically drives central spontaneous glutamate release. The Journal of neuroscience : the official journal of the Society for Neuroscience 30:14470-14475.
Skerry TM, Genever PG (2001) Glutamate signalling in non-neuronal tissues. Trends in pharmacological sciences 22:174-181.
Sluka KA, Dougherty PM, Sorkin LS, Willis WD, Westlund KN (1992) Neural changes in acute arthritis in monkeys. III. Changes in substance P, calcitonin gene-related peptide and glutamate in the dorsal horn of the spinal cord. Brain research Brain research reviews 17:29-38.
Sluka KA, Westlund KN (1993) Spinal cord amino acid release and content in an arthritis model: the effects of pretreatment with non-NMDA, NMDA, and NK1 receptor antagonists. Brain research 627:89-103.
Tsuda M, Koizumi S, Kita A, Shigemoto Y, Ueno S, Inoue K (2000) Mechanical allodynia caused by intraplantar injection of P2X receptor agonist in rats: involvement of heteromeric P2X2/3 receptor signaling in capsaicin-insensitive primary afferent neurons. The Journal of neuroscience : the official journal of the Society for Neuroscience 20:RC90.
Waagepetersen HS, Qu H, Sonnewald U, Shimamoto K, Schousboe A (2005) Role of glutamine and neuronal glutamate uptake in glutamate homeostasis and synthesis during vesicular release in cultured glutamatergic neurons. Neurochemistry international 47:92-102.
Wheeler DD, Boyarsky LL, Brooks WH (1966) The release of amino acids from nerve during stimulation. Journal of cellular physiology 67:141-147.
Woolf CJ, Ma Q (2007) Nociceptors--noxious stimulus detectors. Neuron 55:353-364.
Yang X, Yang HB, Xie QJ, Liu XH, Hu XD (2009) Peripheral inflammation increased the synaptic expression of NMDA receptors in spinal dorsal horn. Pain 144:162-169.
Zahn PK, Sluka KA, Brennan TJ (2002) Excitatory amino acid release in the spinal cord caused by plantar incision in the rat. Pain 100:65-76.
Zhou S, Bonasera L, Carlton SM (1996) Peripheral administration of NMDA, AMPA or KA results in pain behaviors in rats. Neuroreport 7:895-900.

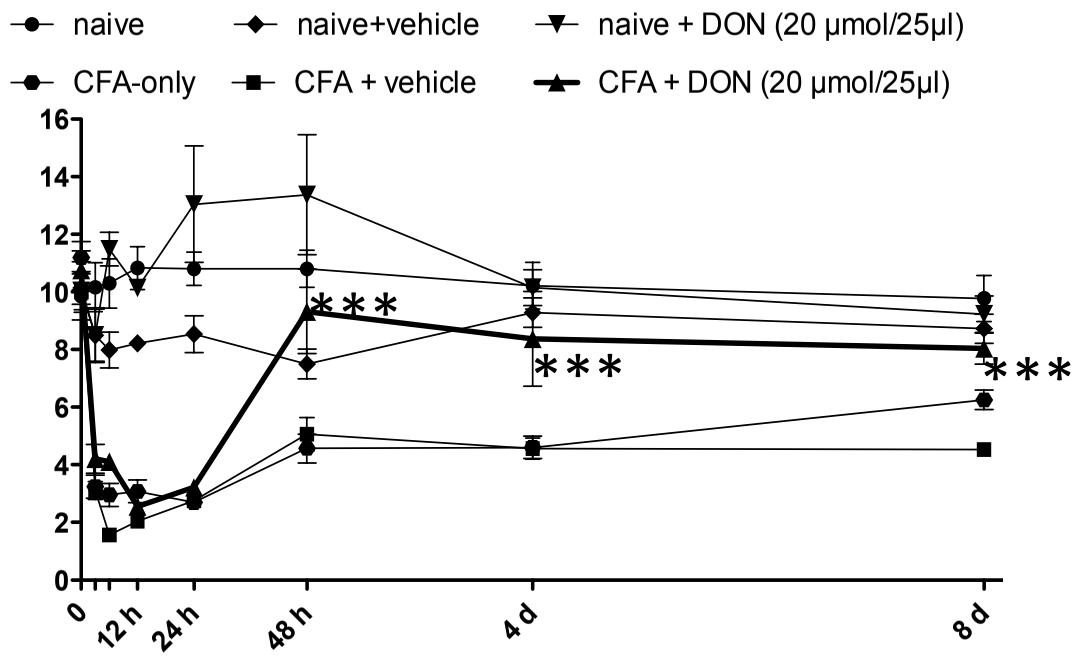
Figure 1

A.


naive + vehicle
naive + DON (20 µmol/25µl)
CFA-only
CFA + vehicle
CFA + DON (20 µmol/25µl)
paw thickness (mm)
11
10
9
8
7
6
***
***
***
***
-12 h
0
12 h
24 h
48 h
4 d
8 d

B.

thermal withdrawal



C.

paw pressure force (mN)

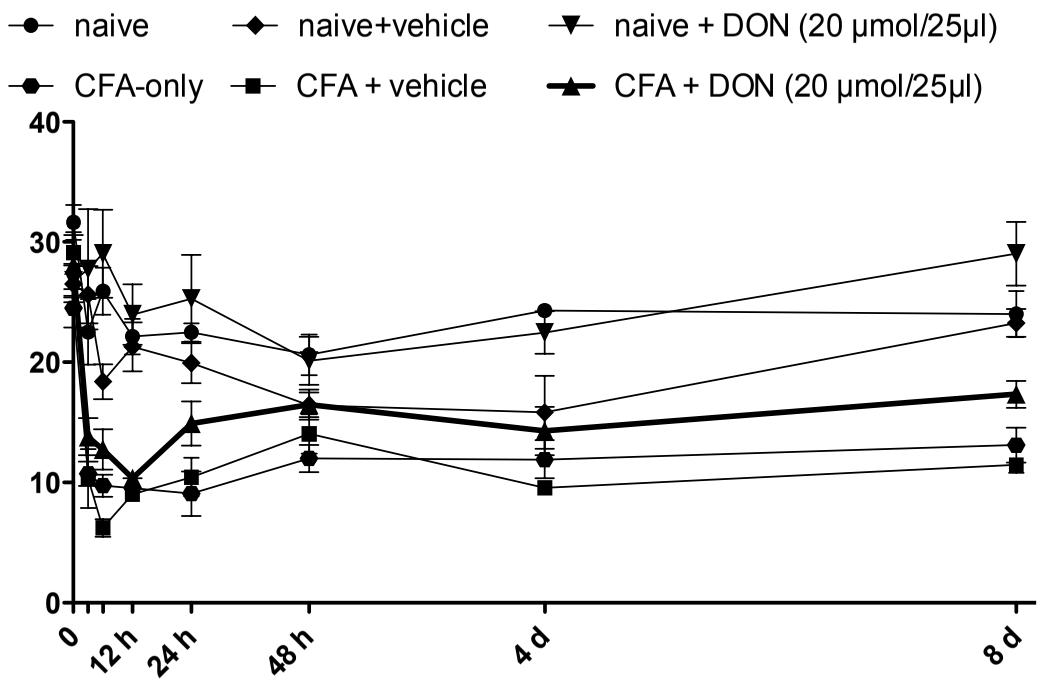
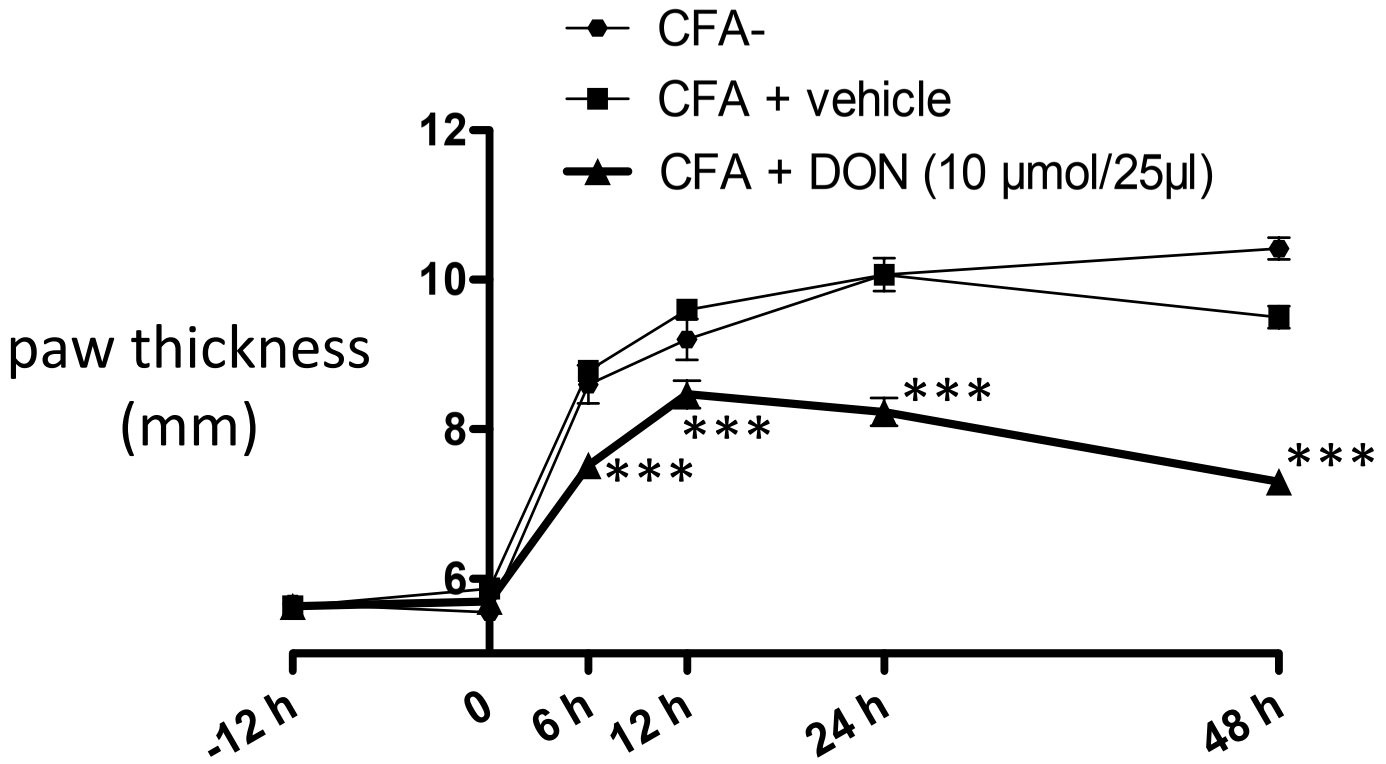


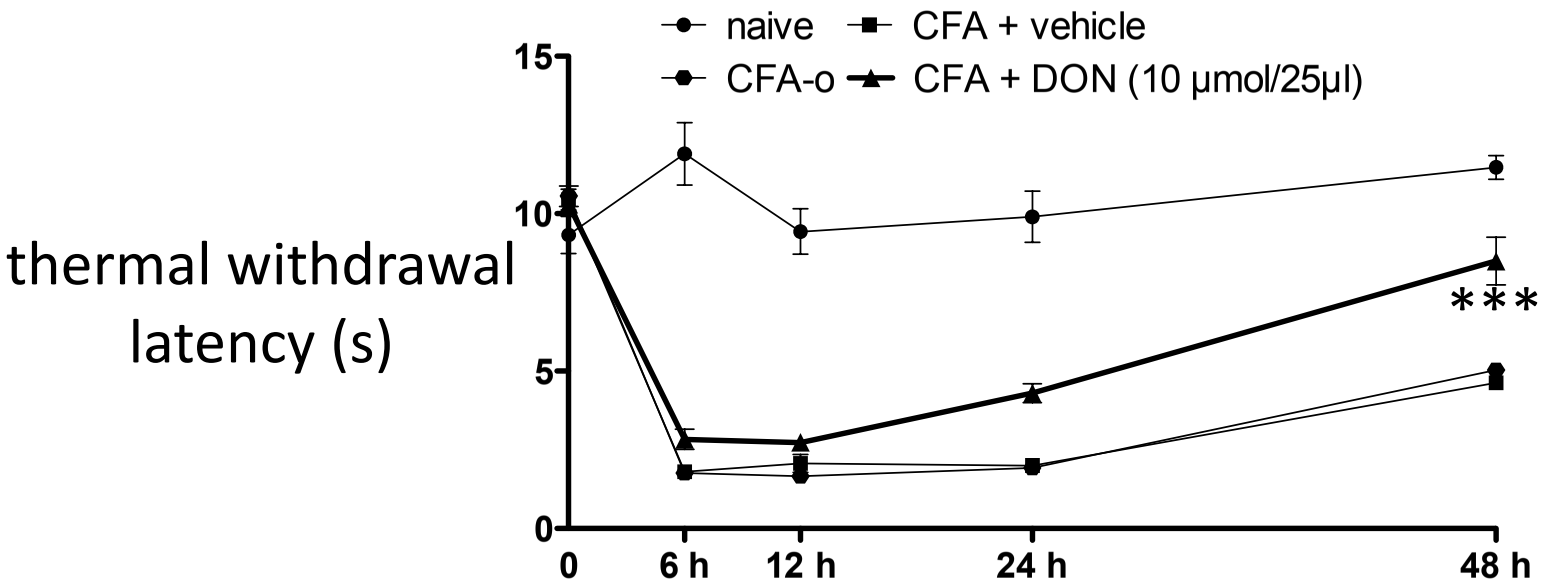
Figure 1 Effects of DON (20μmol/25μl) application at the onset of unilateral adjuvant-induced arthritis (AIA) on edema (A), thermal hyperalgesia (B), mechanical allodynia (C). Thickness of the paw (mm, A), thermal withdrawal latency (s, B) and paw pressure force (mN, C) were plotted against the time course in hours (h) and days (d). The baseline was evaluated prior to any injection, and 20μmol/25μl DON was administered subcutaneously twice at 12 h and 30 min before the CFA injection. Note that DON application significantly attenuated the paw swelling as early as 6 hour after inflammation, and it also reversed thermal hyperalgesia at 48 hour and lasts at least till 8 days after the onset of AIA. Decreased threshold in paw pressure test was observed following injection of CFA in CFA-only, CFA+vehicle and CFA+DON groups from 3 h to 8 days of AIA. No statistic difference was detected in paw pressure force between CFA-only and CFA+DON. The results were mean expressed as the mean ± S.E.M. with six rats per treatment. *P < 0.05, **p < 0.01, ***p < 0.001 CFA+DON group significantly different from CFA-only group. Two-way ANOVA followed by Dunnett's post-hoc test was performed at each time point.

Figure 2

A.


CFA-
CFA + vehicle
CFA + DON (10 µmol/25µl)
paw thickness (mm)
12
10
8
6
***
***
***
***
-12 h
0
6 h
12 h
24 h
48 h

B.


naive
CFA + vehicle
CFA-o
CFA + DON (10 µmol/25µl)
thermal withdrawal latency (s)
15
10
5
0
0
6 h
12 h
24 h
48 h
***

C.

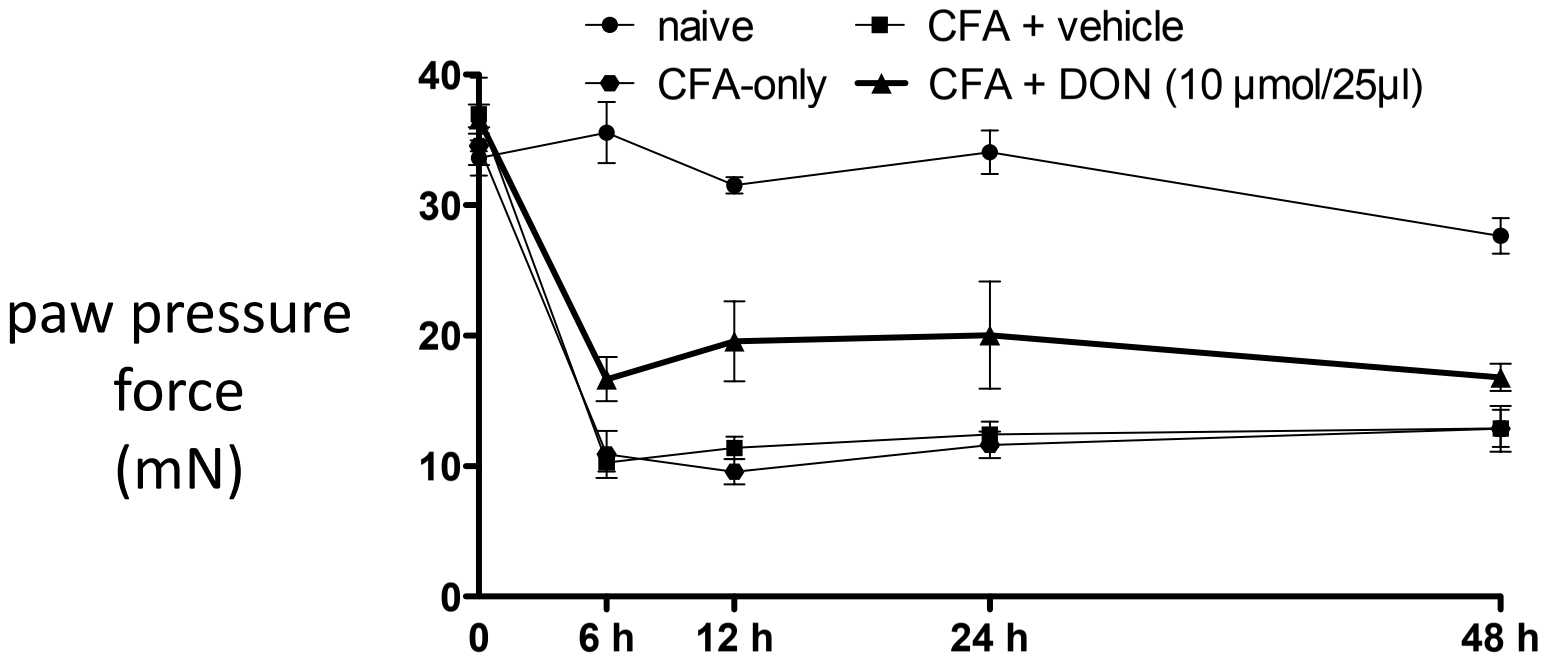
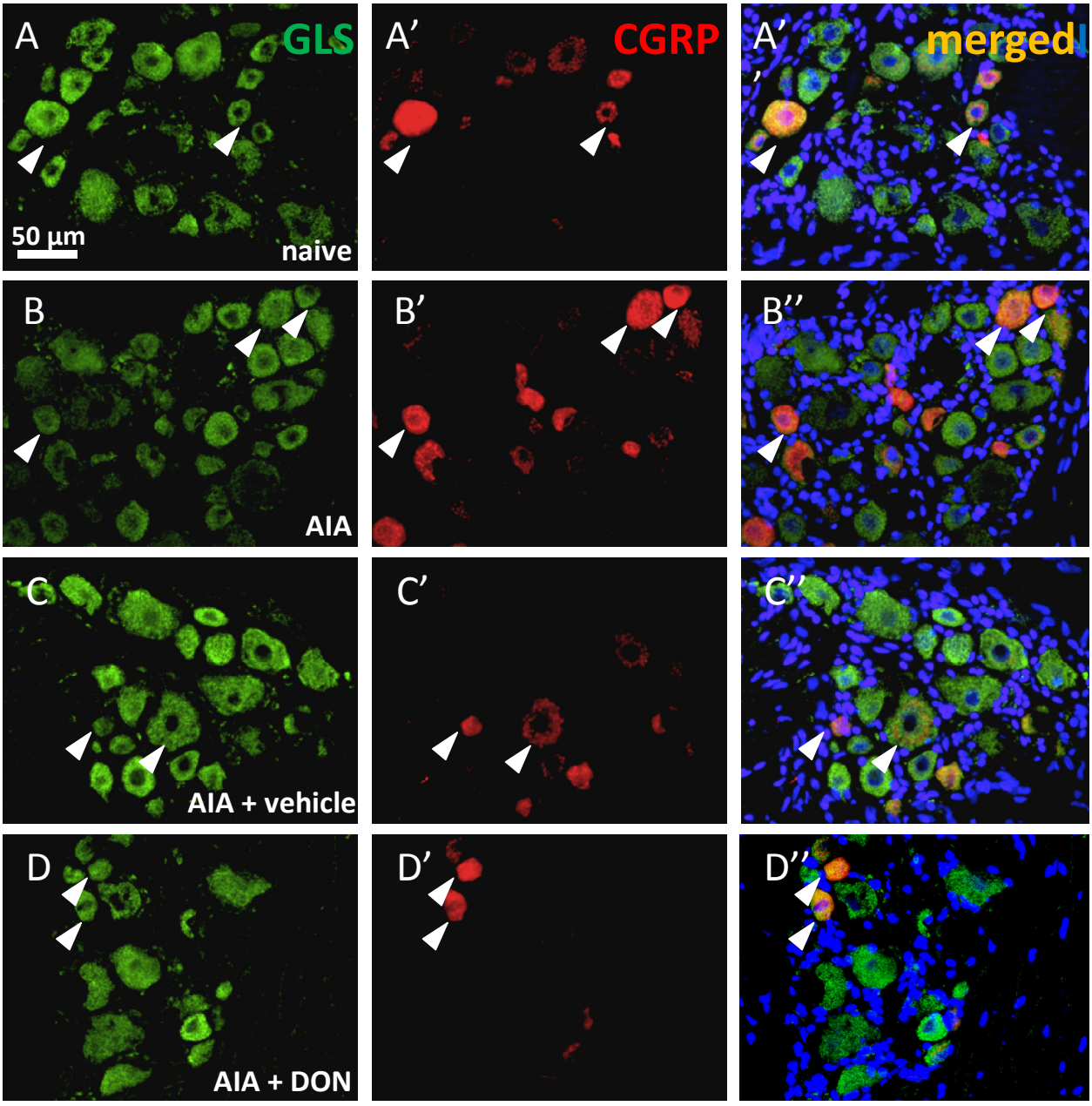

naive
CFA + vehicle
CFA-only
CFA + DON (10 µmol/25µl)
paw pressure force (mN)
40
30
20
10
0
0
6 h
12 h
24 h
48 h

Figure 2 Effects of DON (10μmol/25μl) application at the onset of unilateral adjuvant-induced arthritis (AIA) on and edema (A), thermal hyperalgesia (B) and mechanical allodynia (C). Thickness of the paw (A), thermal withdrawal latency (B) and paw pressure forces (C) were plotted against the time course in hours (h) and days (d). The baseline was evaluated prior to any injection, and 10μmol/25μl DON was administered subcutaneously twice at 12 h and 30 min before the CFA injection. Pre-treatment with 10μmol/25μl of DON achieved inhibitory effects on paw edema from 6 h to 48 h AIA (A) and thermal hyperalgesia were significantly reversed at 48 h (B). Decreased threshold in paw pressure test was observed following injection of CFA in CFA-only, CFA+vehicle and CFA+DON groups from 6 h to 48 h of AIA. No differences were observed in mechanical allodynia with CFA + DON treatment at 10μmol/25μl. The results were mean expressed as the mean ± S.E.M. with three rats per treatment. *$P < 0.05$, **$p < 0.01$, ***$p < 0.001$ significantly different from vehicle-treated in AIA rats. One-way ANOVA followed by Dunnett's post-hoc test was performed at each time point between the four experimental groups.

Figure 3


A
GLS
A'
CGRP
A''
merged
50 µm
naive
B
B'
B''
AIA
C
C'
C''
AIA + vehicle
D
D'
D''
AIA + DON

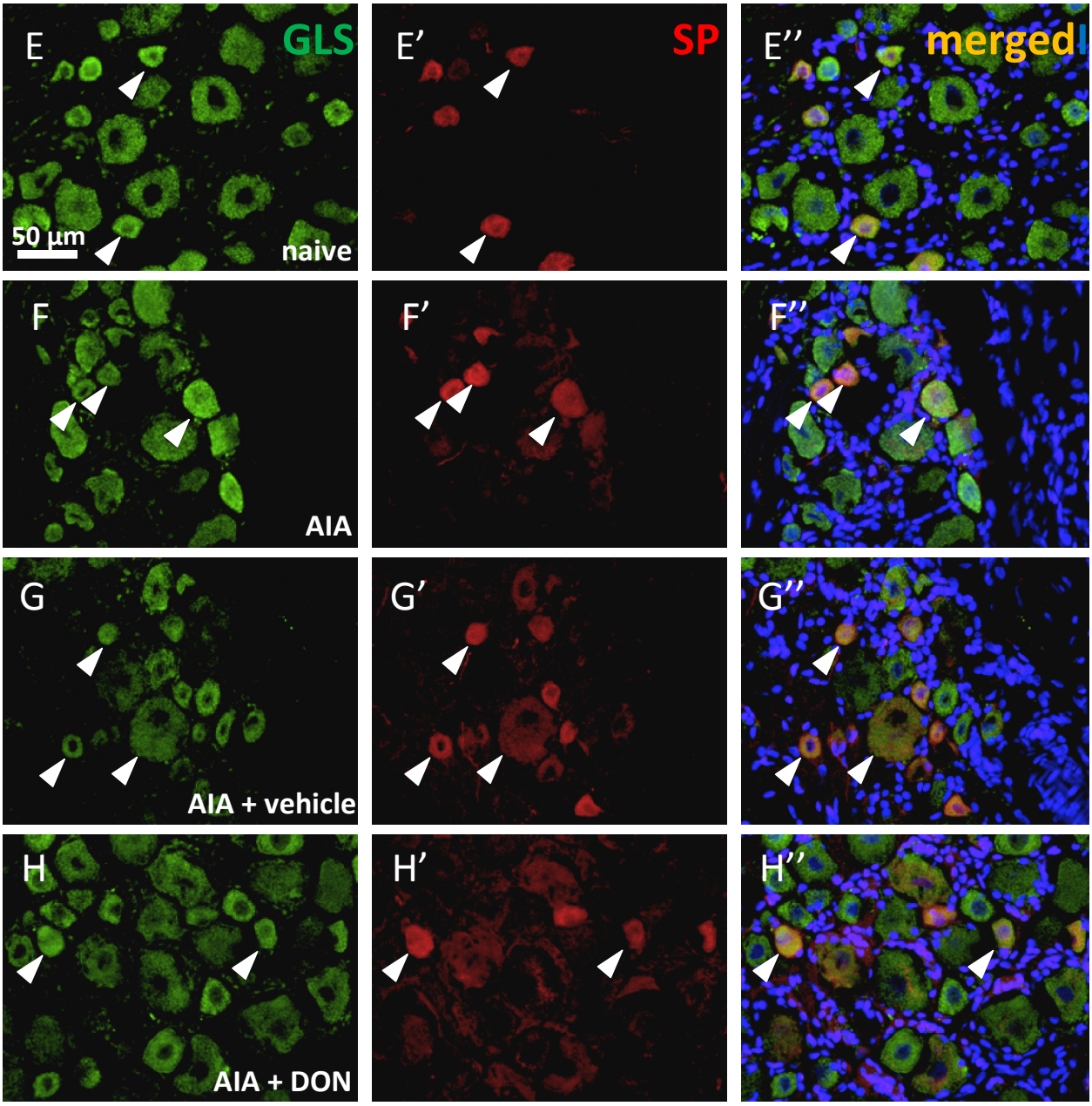
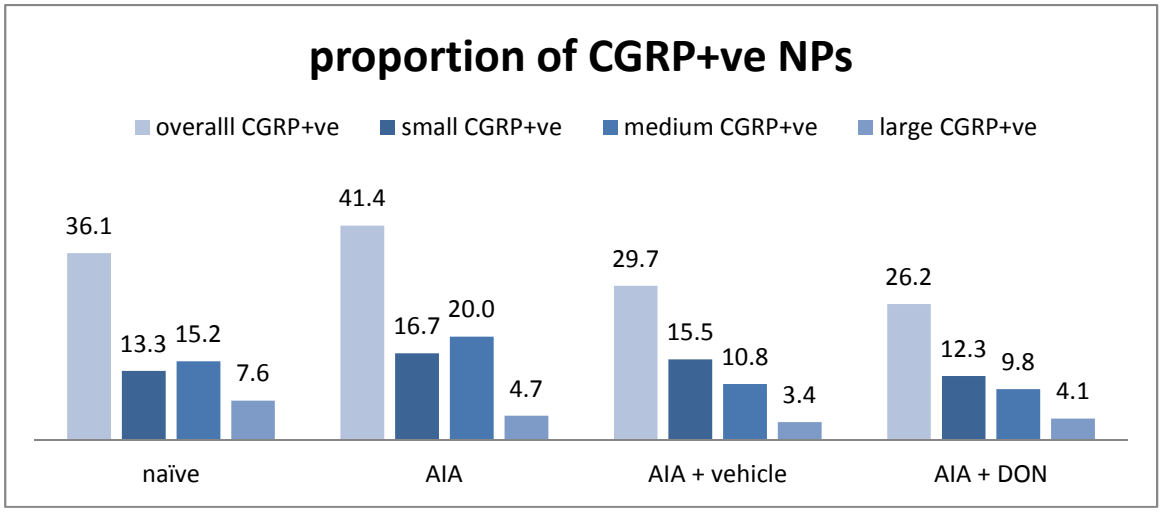


Figure 3. Representative photomicrographs of DRG sections at 48 hour of adjuvant-induced arthritis (AIA) with vehicle or DON (10µmol/25µl) to demonstrate the qualitative effect of DON on immunoreactivity of GLS-ir co-labeled with CGRP **(A-D")** and SP **(E-H")**. Arrowheads indicate DRG neurons that co-express GLS and CGRP or SP in the merged images **(A''-H')**. The quantitative results are obtained and compared by image analysis. Blue = DAPI. Scale bar (**A**) = 50 µm. The scale bar was applied to all the photomicrographs.

Figure 4

A.


proportion of CGRP+ve NPs
overalll CGRP+ve
small CGRP+ve
medium CGRP+ve
large CGRP+ve
36.1
13.3
15.2
7.6
41.4
16.7
20.0
4.7
29.7
15.5
10.8
3.4
26.2
12.3
9.8
4.1
naïve
AIA
AIA + vehicle
AIA + DON

B

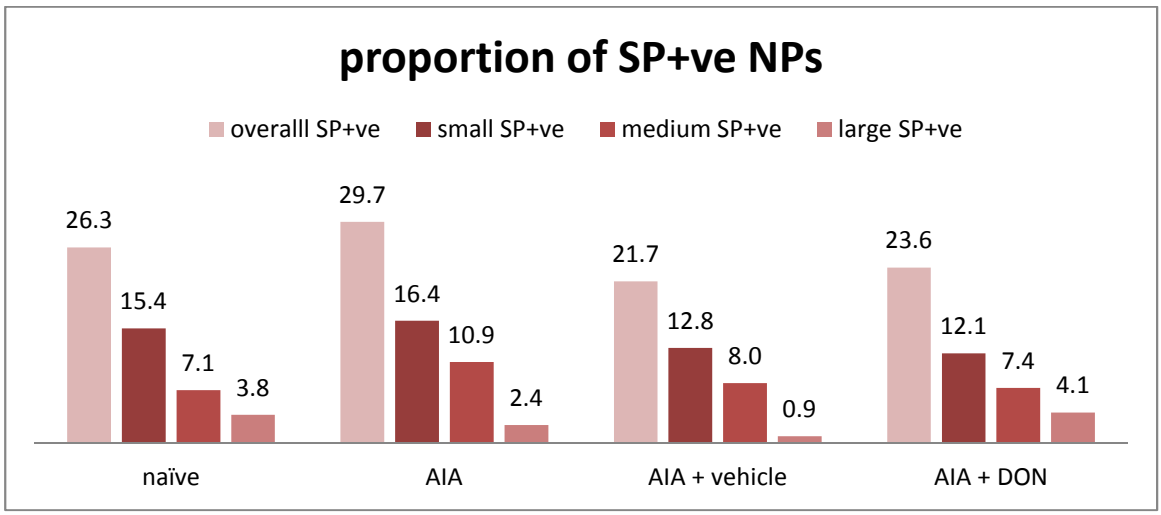

proportion of SP+ve NPs
overalll SP+ve
small SP+ve
medium SP+ve
large SP+ve
26.3
15.4
7.1
3.8
29.7
16.4
10.9
2.4
21.7
12.8
8.0
0.9
23.6
12.1
7.4
4.1
naïve
AIA
AIA + vehicle
AIA + DON

Figure 4. The effect of DON (10μmol/25μl) administration on the proportion of CGRP+ve **(A)** and SP+ve **(B)** at 48 hours following the initiation of adjuvant-induced arthritis (AIA). AIA produces an acute increase in the proportion of CGRP+ve neurons at 48 hours (~5%). DON application, not the vehicle, significantly inhibited the increased proportion of CGRP+ve NPs, especially in small- to medium-sized DRG NPs. **(B)** No change occurred in the proportion of SP+ve NPs was observed.

Figure 5

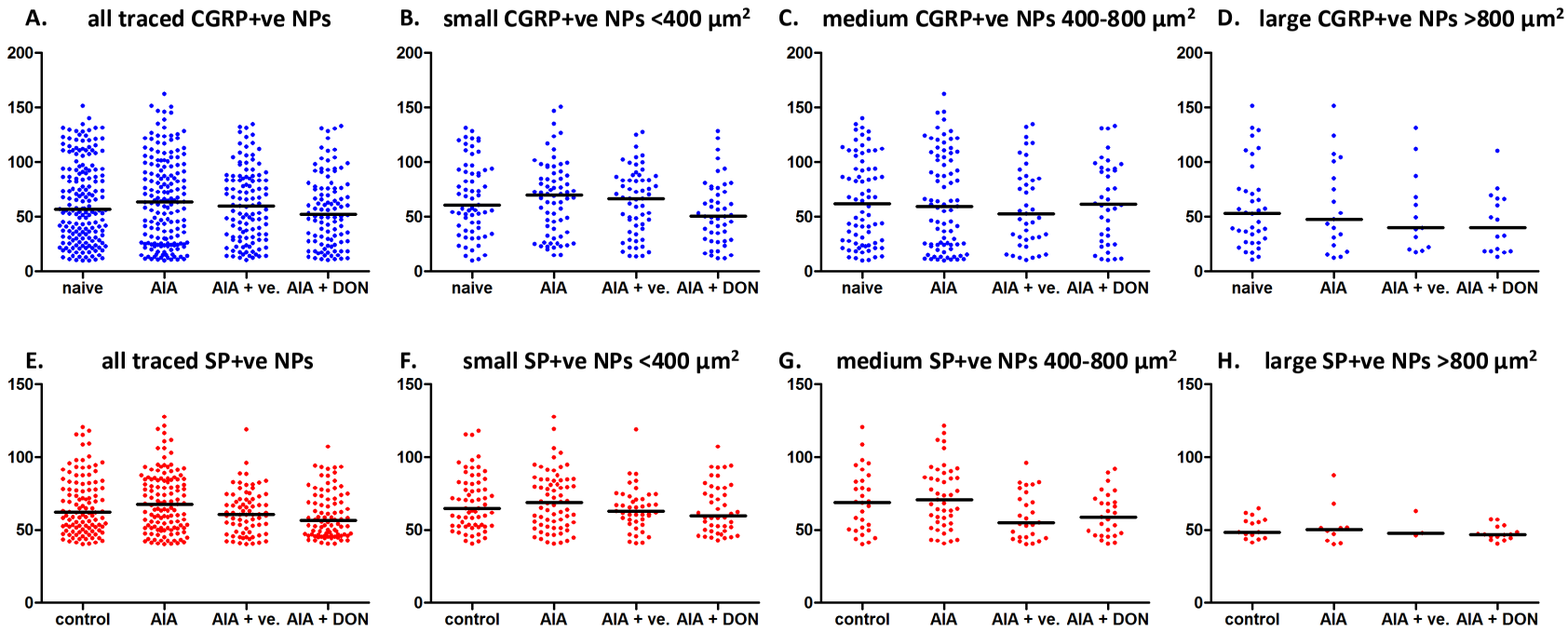

A. all traced CGRP+ve NPs
B. small CGRP+ve NPs <400 µm²
C. medium CGRP+ve NPs 400-800 µm²
D. large CGRP+ve NPs >800 µm²
E. all traced SP+ve NPs
F. small SP+ve NPs <400 µm²
G. medium SP+ve NPs 400-800 µm²
H. large SP+ve NPs >800 µm²
naive
control
AIA
AIA + ve.
AIA + DON
0
50
100
150
200

Figure 5. MGI of CGRP+ve **(A-D)** and SP+ve **(E-F)** at 48h following adjuvant-induced arthritis (AIA). No significant alteration is observed in CGRP- and SP-MGI in DRG NPs after CFA-injection, and DON application does not affect the neuropeptide-ir in the DRG neuronal cell bodies. Kruskal-Wallis nonparametric one-way ANOVA was performed followed by Dunn *posthoc* test.

Figure 6

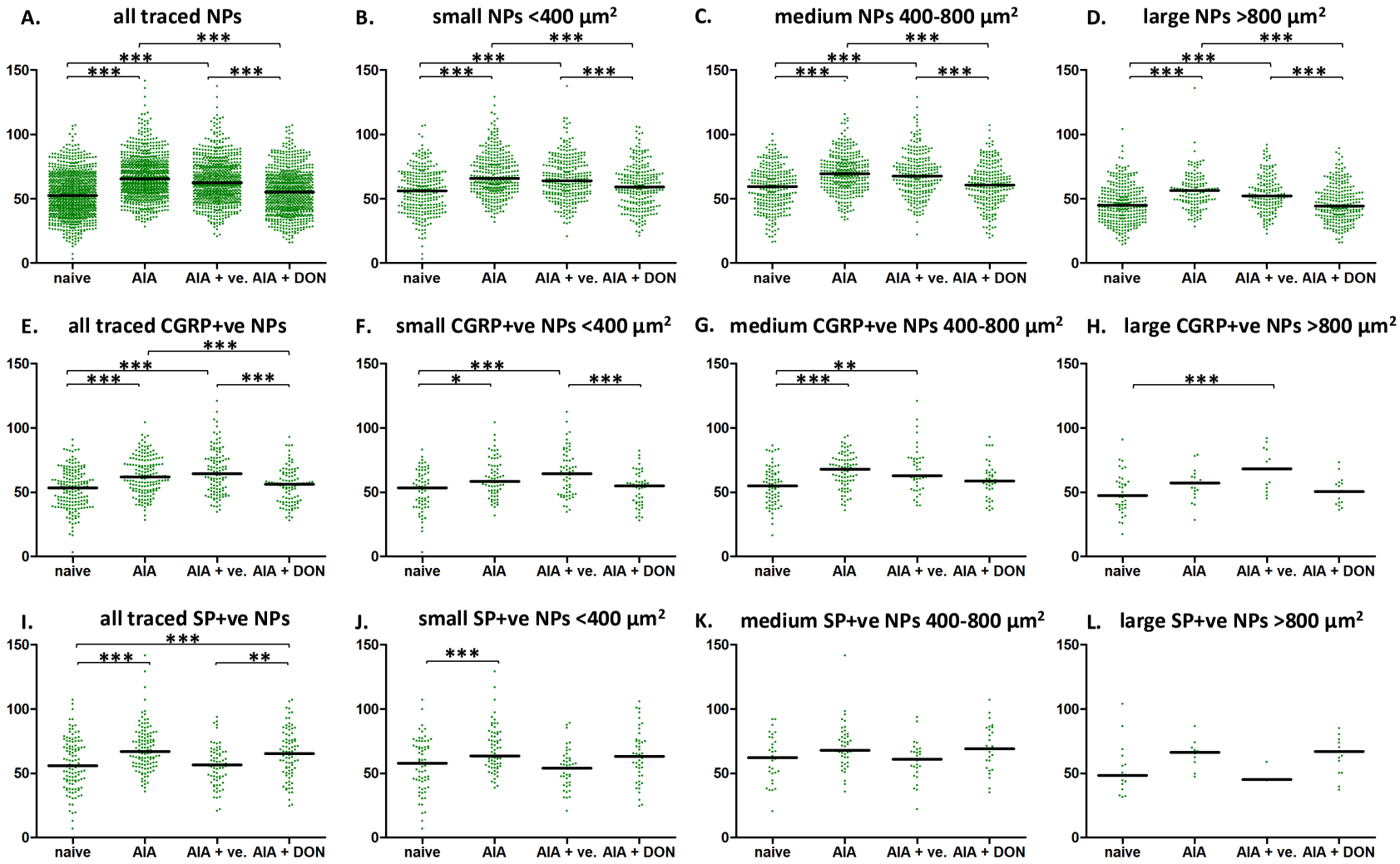

A. all traced NPs
B. small NPs <400 µm²
C. medium NPs 400-800 µm²
D. large NPs >800 µm²
E. all traced CGRP+ve NPs
F. small CGRP+ve NPs <400 µm²
G. medium CGRP+ve NPs 400-800 µm²
H. large CGRP+ve NPs >800 µm²
I. all traced SP+ve NPs
J. small SP+ve NPs <400 µm²
K. medium SP+ve NPs 400-800 µm²
L. large SP+ve NPs >800 µm²
naive
AIA
AIA + ve.
AIA + DON
0
50
100
150
***
**
*

Figure 6. Effects of DON application on GLS-MGI (GLS-MGI) in all traced neuronal profiles (NPs) **(A-D)**, CGRP+ve **(E-H)** and SP+ve **(I-L)** NPs at 48h following adjuvant-induced arthritis (AIA). **(A-D)** AIA causes an acute increase of GLS-MGI in all the traced NPs **(A)** across different sizes **(B-D)**. Application of DON, but not the saline vehicle, reverses the elevated GLS-MGI induced by CFA-induced inflammation **(A-D)**. In CGRP- **(E-H)** and SP- **(I-L)** labeled NPs, and the increase of GLS-MGI is observed in small- **(F, J)**, and some of medium-sized **(G, K)** NPs. This increase in GLS-MGI was reversed by application of DON. Kruskal-Wallis nonparametric one-way ANOVA was performed followed by Dunn posthoc test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

Discussion

Glutamate is the major neurotransmitter utilized by primary sensory neurons at both central and peripheral nerve terminals (Miller et al., 2011). Peripherally released glutamate also serves as a sensitizer that causes increased nociceptive transmission by overstimulation of the glutamate receptor located on the nociceptive nerve terminals (Zhou et al., 1996, Coggeshall and Carlton, 1998, Carlton and Coggeshall, 1999, Carlton, 2001, Du et al., 2006). This increased nociceptive transmission is further conveyed to the central nervous system and interpreted as increased sensation of pain. Use of various glutamate receptor antagonists at central and peripheral terminals successfully alleviates nociception in various acute and chronic inflammatory pain models (Omote et al., 1998, Carlton and Coggeshall, 1999, Carlton, 2001, Du et al., 2006). Limited studies, however, have been done on the role of glutamate metabolism on nociception and sensitization regarding primary afferent neurons. The regulation of glutamate metabolism in response to peripheral inflammation has been the major interest of this laboratory, with the special interest in glutaminase, the neuronal synthetic enzyme that converts glutamine to glutamate. The principal goal of the current studies is to further explore the role of dorsal root ganglion glutaminase in acute and chronic inflammatory pain. This goal is further divided into three aims to stress the contribution of glutaminase to inflammatory pain.

Previous studies from our laboratory have shown that glutaminase expression and enzyme

activity elevate in the neuronal cell bodies and nerve terminals in the inflamed skin during the chronic phase of rat adjuvant-induced arthritis (AIA) model. The elevated glutaminase at the site of inflammation indicates that the altered amount of glutaminase is likely to be present earlier in the neuronal cell bodies in order to be transported to the inflamed site prior to the chronic phase of AIA. Less is known, however, about the response of glutaminase during the acute phase of inflammation and its role in acute inflammatory pain. To better understand the temporal alteration of glutaminase in response to peripheral inflammation, in Chapter II we extended our study by evaluating the alterations of glutaminase levels during both the acute (0-4 days) and chronic (4-8 days) phases of AIA. It was found that glutaminase in DRG neuronal cell bodies was significantly elevated at 1 and 2 days of AIA, followed by a gradual decrease below baseline level at 4 day AIA with another trend of elevation from 4 to 8 days of AIA. These results provide further evidence that the glutaminase expression in the DRG is temporally regulated during the acute and chronic phases of peripheral inflammation. It indicates that glutaminase expression in the DRG is affected by various cellular and molecular mechanisms and contributes to different stages of inflammatory pain.

DRG neurons can be further classified by their morphology, functions and neurochemical characteristics (Lawson, 2002, Woolf and Ma, 2007), and these properties are generally correlated. It is accepted that large sized neurons have large diameter axons and fast conduction velocity, and they mainly transduce sensations including vibration, fine touch, and proprioception; small sized neurons have small diameter axons and slow conduction velocity, and they convey sensations such as pain and temperature. With the knowledge that DRG neurons are heterogenous, it is necessary to study the function of DRG neurons by dividing them into subpopulations. Most of the current techniques measure the relative total protein expression level using tissue homogenates, such as radioimmuoassays, Western blots, and enzyme-linked immunosorbent assays. These techniques have limitations when trying to study the alteration of

protein expression in subpopulations of cells. In Chapter II, we combined double-labeling immunohistochemistry (IHC) and image analysis techniques in order to further evaluate the alteration of glutaminase in nociceptive neurons. By co-labeling with putative markers for peptide-containing nociceptive neurons and dividing the traced neuronal profiles by their cross-sectional area, it was observed that elevated glutaminase is found in small- and medium-sized peptide-containing DRG neurons, which potentially are nociceptors. These results provide further understanding of glutaminase by putting it in context with the peptide-containing DRG neurons and characterizing the neurochemical alteration of this subpopulation of DRG neurons.

In order to study the properties of DRG in subgroups of neurons, great efforts have been made to correlate the sensory modalities with the neurochemical components. In these types of studies, it is crucial to determine the immunoreactivity (IR) of the marker as IR-positive or - negative. There are two general methods to do so. The first method is to use a trained scientist that is blind to the study to determine whether a neuron is considered IR-positive. This method is not able to measure the accurate level of fluorescence of the marker, nor is it suitable for processing large quantities of data. The second method is using normalized immunofluorescent intensity of each neuronal profile and the threshold for the marker is arbitrarily determined by the percentage of relative intensity (Fang et al., 2005, Fang et al., 2006, Hoffman et al., 2010). The later method is more objective than the former one and is employed in many studies of DRG neurons using image analysis technique. In our studies, we proposed a modified approach to determine IR-threshold based on the second method. It is described in details in the section of Chapter II – Method. We believe that our approach provides a more objective way to determine the IR-threshold for any fluorophore-conjugated molecular probe. It will benefit those researchers who are concerned with both cyto-/histo-chemical contents and cell/tissue morphology in various cell types.

In Chapter II, an acute elevation of glutaminase in the DRG neuronal cell bodies was observed and the increased glutaminase also has been observed in the inflamed skin at the chronic phase of AIA. It is reasonable to assume that elevated glutaminase is transported from the neuronal cell bodies to the nerve terminal. No studies, however, have been done to evaluate glutaminase levels in the peripheral nerve in response to peripheral inflammation. In order to test our assumption, in Chapter III the accumulation of glutaminase was studied in response to peripheral inflammation in the sciatic nerve, which contains the peripheral branches of DRG neurons that innervate the hindpaw skin. The alteration of DRG glutaminase was initiated using the same animal modal and protocol in Chapter II and a ligature placed at the sciatic nerve by surgery at different time points after the initiation of AIA. The accumulation of IR then was evaluated qualitatively and quantitatively using fluorescent microscopy and image analysis techniques. It was observed that a significant accumulation of elevated glutaminase in the sciatic nerve at 2 days following AIA, which is in accordance with the peak glutaminase elevation in the neuronal cell bodies at day 1. These results provide support for our assumption that the elevated glutaminase in the neuronal cell bodies is transported in the peripheral nerve and delivered to the nerve terminals around the site of inflammation, where it contributes to excess glutamate production. Results of Chapter III also indicate that when studying the cellular change of a molecule, it is necessary to consider and integrate temporal and spatial alterations to build a four-dimensional view and better target the molecule of interest *in vivo*.

Thus far, Chapter II and III have demonstrated the elevation of glutaminase in response to inflammation. This newly synthesized glutaminase is “elicited” from the neuronal cell body as the result of inflammation. It has been shown that peripheral inhibition of glutaminase at 3 days of AIA with a glutaminase inhibitor produces a powerful inhibitory effect on chronic inflammatory pain (Miller et al., 2010). These results indicate that elevated glutaminase produced in the cell body and transported in the peripheral target will contribute to acute and inflammatory pain by

producing more glutamate in nerve terminals for sensitizing the inflammatory site. In Chapter IV, we wanted to evaluate the role of endogenous or tonic glutaminase and glutamate that pre-exist at the nerve terminal in contributing to acute and chronic inflammatory pain behavior, as well as their contribution to the "elicited" alteration of glutaminase in the DRG neuronal cell bodies. By pre-blocking glutamate production with the glutaminase inhibitor, 6-diazo-5-oxo-L-norleucine (DON), it was observed that an acute inhibitory effect on paw edema from immediately after the AIA initiation to 48 hours AIA and a long-term suppression on thermal hyperalgesia from 48 hours of AIA at least to 8 days of AIA. Furthermore, the elevation of glutaminase-IR in DRG neuronal cell bodies was attenuated. These results indicate that pre-depletion of glutamate can produce inhibitory effects on 1) the generation of edema and 2) the development and maintenance of acute and chronic inflammatory pain. It indicates that the tonic or elevated glutamate during the acute phase of inflammation has an important role in the development of chronic inflammatory pain. If left untreated, some acute pains can develop into chronic pain with prolonged suffering. Our studies suggest that treatment targeting early glutamate elevation after acute inflammation/trauma/injury may be beneficial for effective alleviation of pain that often progresses to chronic stages. Our approach is one of the few studies that demonstrate the role of glutamate as a sensitizer in nociception at the peripheral nerve terminal by inhibiting peripheral glutamate production. Compared to the studies using glutamate antagonists, inhibition of glutaminase provides more powerful and long-term analgesic effects, which makes peripheral glutaminase a promising target for development of novel analgesic drugs.

Summary

In Chapter II and III, we characterized the temporal and spatial responses of dorsal root ganglion glutaminase to peripheral inflammation. Significant elevation of glutaminase was observed in subpopulations of DRG neurons that were labeled with nociception-related neurochemical markers in small- to medium-sized neuronal cell bodies. These results indicate that after

inflammation, elevated glutaminase is synthesized in the neuronal cell bodies and then transported to the peripheral nerve terminals around the inflamed site, where glutamate is produced and released contributing to peripheral sensitization. In Chapter IV, we further explored the role of basal glutaminase and glutamate in contributing to acute and chronic inflammatory pain. These studies help us to understand the role of glutamate as a sensitizer at the peripheral nerve terminal from the perspective of the glutamate metabolism. It also supports the notion that early treatment that targets the acute glutamate elevation at the peripheral terminal may prevent the development of chronic pain and/or provide more effective alleviation if the pain continues into the chronic phase with the pathology. This can be extremely beneficial to patients who suffer from sport-related injury or trauma, where immediate medical attention and care is usually more available compared to chronic diseases.

With further understanding of the role of dorsal root ganglion glutaminase in acute and chronic inflammatory pain, it is likely to predict that glutaminase and glutamate metabolism can be new potential targets for novel analgesic drugs.

Future directions

It was mentioned previously that correlating neurochemical components with their sensory modalities is crucial for studying the primary sensory neurons. No perfect neurochemical markers, however, have been identified to classify the sensory modalities of the labeled neurons. This means there always are overlaps in the expression of neurochemical markers and the functions among DRG neurons. Possibly there are new neurochemical markers to be discovered for specific identification of DRG neurons or possibly the neurochemical components of the DRG neurons are like a coding system. It is not defined by a simple “positive-negative” or “true-false” style. Instead, DRG neurons may use the combined expression pattern of numerous neurochemical markers to code their function and response when challenged. More efficient and

powerful analyzing methods, therefore, may be needed to fully understand the expression pattern in DRG neuronal subpopulations and as a total population. Thus far, flow cytometry has been a powerful technique applied in clinical and in basic research. In most cases, flow cytometry allows the detection and separation of cells with multiple fluorophore-conjugated probes on various molecular levels, such as protein, mRNA, mitochondria membrane potential and etc, while preserving the cell morphology. The advantages of flow cytometry meet the requirements for our studies and will be a powerful technique to fulfill the purposes of quantification and preparation for further analysis, such as cell culture, electrophysiology, microarray and etc. Over the past year, I have been working on dissociation of DRG neurons and separation using flow cytometry with Dr. Kent Teague, University of Oklahoma Tulsa. We will continue this project in the near future to verify the results from the present dissertation.

In the current studies, the major focus was characterization of the alteration of glutaminase after inflammation in the DRG neurons. More studies, however, need to be done to reveal the cellular and molecular mechanisms that contribute to glutaminase alterations in DRG neurons. Neurotrophin trafficking and cell signaling will be studies that should be explored in the future. Besides the changes that occur in peripheral nerve terminals, alteration of glutamate metabolism and signaling events at central terminals of primary afferents have significant contribution to chronic and persistent pain. This area, too, will need to be examined to obtain better understanding of acute and chronic pain. Lastly, a concept that is gaining acceptance is that pain is not only a symptom that comes with the disease, but also a disease itself that requires more attention for management. These results indicate that glutaminase may be a potential therapeutic target for novel analgesic drugs to address pain 'disease' regardless of the disease source.

Reference

Carlton SM (2001) Peripheral excitatory amino acids. Current opinion in pharmacology 1:52-56.

Carlton SM, Coggeshall RE (1999) Inflammation-induced changes in peripheral glutamate receptor populations. Brain research 820:63-70.

Coggeshall RE, Carlton SM (1998) Ultrastructural analysis of NMDA, AMPA, and kainate receptors on unmyelinated and myelinated axons in the periphery. The Journal of comparative neurology 391:78-86.

Du J, Zhou S, Carlton SM (2006) Kainate-induced excitation and sensitization of nociceptors in normal and inflamed rat glabrous skin. Neuroscience 137:999-1013.

Fang X, Djouhri L, McMullan S, Berry C, Okuse K, Waxman SG, Lawson SN (2005) trkA is expressed in nociceptive neurons and influences electrophysiological properties via Nav1.8 expression in rapidly conducting nociceptors. The Journal of neuroscience : the official journal of the Society for Neuroscience 25:4868-4878.

Fang X, Djouhri L, McMullan S, Berry C, Waxman SG, Okuse K, Lawson SN (2006) Intense isolectin-B4 binding in rat dorsal root ganglion neurons distinguishes C-fiber nociceptors with broad action potentials and high Nav1.9 expression. The Journal of neuroscience : the official journal of the Society for Neuroscience 26:7281-7292.

Hoffman EM, Schechter R, Miller KE (2010) Fixative composition alters distributions of immunoreactivity for glutaminase and two markers of nociceptive neurons, Nav1.8 and TRPV1, in the rat dorsal root ganglion. J Histochem Cytochem 58:329-344.

Lawson SN (2002) Phenotype and function of somatic primary afferent nociceptive neurones with C-, Adelta- or Aalpha/beta-fibres. Exp Physiol 87:239-244.

Miller KE, Herzog B, Sutharshan M (2010) 6-Diazo-5-Oxo-L-Norleucine: A Glutaminase Inhibitor with Local Analgesic Properties. In: IASP, vol. 4193 Montreal, Qeubec, Canada.

Miller KE, Hoffman EM, Sutharshan M, Schechter R (2011) Glutamate pharmacology and metabolism in peripheral primary afferents: physiological and pathophysiological mechanisms. Pharmacol Ther 130:283-309.

Omote K, Kawamata T, Kawamata M, Namiki A (1998) Formalin-induced release of excitatory amino acids in the skin of the rat hindpaw. Brain research 787:161-164.

Woolf CJ, Ma Q (2007) Nociceptors--noxious stimulus detectors. Neuron 55:353-364.

Zhou S, Bonasera L, Carlton SM (1996) Peripheral administration of NMDA, AMPA or KA results in pain behaviors in rats. Neuroreport 7:895-900.

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