STRESS HORMONE INFLUENCES ON NEURAL AND IMMUNE MECHANISMS OF NEUROPATHIC PAIN

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

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2010

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Abstract

Chronic pain is a leading world health problem. It retards personal and societal productivity, and engenders despair. One person in five suffers the multidimensional consequences of chronic pain. Neuropathic pain is a particularly debilitating subtype of chronic pain that derives from damage to the somatosensory nervous system. Tens of millions globally are affected by neuropathic pain. Many experience persistent, unprovoked burning and shock-like pain for which we currently have minimally effective treatments. There are numerous comorbidities associated with painful conditions, including stress, depression and anxiety. Stress increases the severity of persistent pain and spurs episodic pain. For conditions that can lead to neuropathic pain, (e.g., SCI, stroke, HIV, cancer), stress may hasten the onset and/or increase the severity of pain.

Based on anecdotal and published reports, stress is a significant contributor to pain; despite awareness of this relationship, mechanism(s) has largely been overlooked. Herein, we address this gap to better understand the role of stress and stress hormones in persistent pain.

To test the hypothesis that stress exacerbates the development of neuropathic pain, acute restraint stress was applied immediately prior to peripheral nerve injury and subsequent pain-like behavior was measured. As reported in humans, stress increased allodynia in mice. Stressors elicit numerous physiological changes that could account for enhanced
pain. Among them, is activation of the hypothalamic-pituitary-adrenal (HPA) axis that stimulates glucocorticoid (GC) release. Cortisol is the primary stress hormone of the GC family, (NB: corticosterone in rodents). The actions of GCs are initiated by binding the glucocorticoid receptor (GR). The density of GR is variable throughout the nervous system and its relative density in somatosensory circuitry was unknown. Remarkably, we observed that GR abundance in dorsal root ganglia (DRG) exceeded the hippocampus, where it is generally considered most abundant. Even in a more heterogeneous tissue as the dorsal spinal cord (including white matter), GR content was comparable to hippocampus. Thus, the potential for GCs to affect pain processing circuitry is considerable.

To determine if stress exerts its effect on pain via GC-GR activity, mice were treated with a GR antagonist prior to stress. Indeed, GR blockade attenuated stress effects on pain-like behavior after nerve injury. Importantly, besides perceived stressors, there are multiple stimuli that induce GC release, including bodily injury, illness, and exercise. Hence, in broad contexts, exposure to GCs may impact pain. To determine if exposure to elevated GCs is sufficient to produce pain enhancement, mice were treated systemically with corticosterone (CORT) prior to nerve injury (in lieu of stress). Like the stressor, this procedure also increased allodynia in mice. Together, these data indicate that stress-induced potentiation of allodynia is at least partially mediated by GR.
Two broad mechanisms contribute to neuropathic pain: neural plasticity and neuroinflammation. Stress and GCs are known to affect measures of both neural plasticity and neuroinflammation, therefore their impact on pain could be through either mechanism or, as our studies suggest, on both. To investigate structural neuron plasticity associated with pain, we measured the axon growth capacity of DRG neurons derived from acutely stressed mice. Compared to no stress, stress increased measures of axon sprouting and elongation. This in vitro sprouting phenotype is generally compared to the type of axon sprouting observed in painful neuromas; whereas, elongating axons are likened to those that support functional regeneration. In effect, it appears that stress stimulated a growth ‘program’ in DRG neurons that simultaneously promotes both unfavorable and favorable axon phenotypes. In subsequent experiments, these effects were observed to be GR, NMDAR, and mTOR dependent. To verify the growth promoting effects of stress in an in vivo regeneration model, we performed a sciatic nerve crush procedure immediately after acute stress. Mice exposed to the stressor demonstrated increased axon regeneration, as well as increased measures of allodynia. Perhaps stress effects on pain, as seen in two neuropathic models, are related to increased structural neuron plasticity. We suspect that the molecular underpinnings of structural plasticity (e.g., NMDAR, mTOR) are shared by measures of functional neuron plasticity, and that together, these act as substrates for stress effects on pain.

Pro-inflammatory cytokines are effectors of both neural plasticity and neuroinflammation mechanisms of neuropathic pain. Cytokines can directly stimulate neurons, or indirectly,
via effects on immune cells. One particular pro-inflammatory cytokine, MIF, is of unique relevance to stress. MIF is induced by stress and GCs, and overrides the anti-inflammatory action of GCs. Its role in pain was unknown, but its constitutive and widespread distribution made it a putative candidate for initiating and propagating injury responses. We hypothesized that stress potentiates pain via MIF. To our surprise, MIF KO mice did not develop pain-like behaviors after nerve injury or CFA-induced paw inflammation, regardless of stress. Likewise, a small molecule inhibitor of MIF reduced allodynia after peripheral nerve injury. Our data demonstrate that MIF plays a critical role in the development and maintenance of persistent pain. To determine if MIF is involved in stress-induced enhancement of pain-like behavior, mice were treated with the MIF inhibitor prior to restraint stress. Stress effects on allodynia were absent in the inhibitor-treated mice, indicating a role for MIF in this context. Supporting its role as a pro-algesic cytokine, intraplantar administration of recombinant MIF (rMIF) alone produced dose-dependent allodynia. Acute restraint stress increased rMIF-induced allodynia. To identify a cellular substrate for MIF, we examined both microglia and neurons. Primary microglia treated with rMIF increased transcript levels of iNOS, IL-1β, and IkBa, indicating the induction of a pro-inflammatory phenotype by rMIF. rMIF also increased measures of both functional and structural sensory neuron plasticity. By targeting both microglial reactivity and neuron plasticity, MIF may play a dual-role in the pathology of persistent pain. These data describe a previously unknown, essential role for MIF in persistent pain.
In summary, we have contributed to the field of neuropathic pain the novel observations that, 1) stress exacerbates the development of neuropathic pain-like behavior, and 2) MIF is essential for persistent pain states. We have identified three putative molecular bases for stress and GC effects on pain: NMDAR, mTOR, and MIF. Of these, MIF is a particularly novel therapeutic target for pain management. We propose that stress and GCs account for some variability in the development and severity of pain in humans.
Dedication

Dedicated to Eric who strengthens and supports me,

and to my parents and Tisha for lifelong inspiration.

To Livi, should you grow up to love science like your big sis.
Acknowledgments

I am fortunate for the support of many, but foremost in my professional development for my advisor, Phillip Popovich. Under your auspices, I have matured into a capable, confident scientist. Thank you sincerely for your dedication, enthusiasm, and guidance. I take with me the critical lesson to ‘philter’ tangential curiosities.

My fortune extends into the laboratory, where I interacted with exceptional, committed scientists and friends. Ming, I owe the most to you. Thank you for your tireless efforts to facilitate my experiments! Krissy, from the start to the end, you were always there for me intellectually and personally. Dan, my M1/M2 partner, thanks for your unique humor. I think I finally ‘got it.’ Asia and Lori, could there be a better corner in the lab?! Akshata, Dave, and Dustin, my peers and friends, I thoroughly enjoyed our time together and will miss your laughter in the lab. On that note, John, I will miss your song and dance! Alicia Z., you were a latecomer, but without question, indispensable to the completion of my projects! Gina, you are an exceptional teammate for the MIF project! Thank you! Rezan, the well-layered night owl, your candor is appreciated. Alicia H. & Yi, it’s been fun having your fresh ideas in the lab. Mel, thank you for your friendship! Wenmin, Amy, Ping, Todd, and Zhen- the real experts- thank you for sharing your talents and skills.
To Dana McTigue, my advisory committee (Drs. Basso, Godbout, and Whitacre), and numerous collaborators, thank you. The investment and encouragement of those, like you, who are not ‘obligated’ to support me is especially meaningful. I appreciate your involvement in my graduate career and look forward to our future interactions. You are all excellent mentors with qualities I hope to emulate in my own work.
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Publications


**Fields of Study**

Major Field: Neuroscience
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List of Abbreviations

5-HT Serotonin
AMPA $\alpha$-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
ATP Adenosine Triphosphate
BDNF Brain Derived Neurotrophic Factor
CaMK Calcium-Calmodulin Dependent Protein Kinase
CCI Chronic Constriction Injury
CCK Cholecystokinin
CCL2/MCP-1 Chemokine (C-C Motif) Ligand 2
CCR2 Chemokine (C-C Motif) Receptor 2
CD Cluster of Differentiation
CGRP Calcitonin Gene Related Peptide
CNS Central Nervous System
CNTF Ciliary Neurotrophic Factor
CORT Corticosterone
CREB cAMP Response Element-binding Protein
CX3CL1 Fractalkine
DRG Dorsal Root Ganglion
EGF Epidermal Growth Factor
ERK Extracellular Signal Related Kinase
<table>
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<tr>
<th>Abbreviation</th>
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<tr>
<td>GC</td>
<td>Glucocorticoid</td>
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<tr>
<td>GR</td>
<td>Glucocorticoid Receptor</td>
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<tr>
<td>HPA</td>
<td>Hypothalamic-Pituitary-Adrenal (Axis)</td>
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<tr>
<td>IASP</td>
<td>International Association for the Study of Pain</td>
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<tr>
<td>Iba-1</td>
<td>Ionized Calcium Binding Protein 1</td>
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<td>IFN-γ</td>
<td>Interferon Gamma</td>
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<td>IL</td>
<td>Interleukin</td>
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<td>mTOR</td>
<td>Mammalian Target of Rapamycin</td>
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<td>NE</td>
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<tr>
<td>NT-3, 4/5</td>
<td>Neurotrophin 3 or 4/5</td>
</tr>
<tr>
<td>p-</td>
<td>Phosphorylated</td>
</tr>
<tr>
<td>P2X</td>
<td>Ligand-gated Ionotropic Purinergic Receptor</td>
</tr>
<tr>
<td>P2Y</td>
<td>G Protein-coupled Purinergic Receptor</td>
</tr>
<tr>
<td>PA</td>
<td>Proportional Area</td>
</tr>
<tr>
<td>PACAP</td>
<td>Pituitary Adenylate Cyclase-activating Peptide</td>
</tr>
<tr>
<td>PAG</td>
<td>Periaqueductal Grey</td>
</tr>
<tr>
<td>PGE2</td>
<td>Prostaglandin E2</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3 Kinase</td>
</tr>
<tr>
<td>PID</td>
<td>Post-Injection Day</td>
</tr>
<tr>
<td>POD</td>
<td>Post-Operative Day</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein Kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
</tr>
<tr>
<td>PSNL</td>
<td>Partial Sciatic Nerve Ligation</td>
</tr>
<tr>
<td>PVN</td>
<td>Paraventricular Nucleus (of the Hypothalamus)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Rap</td>
<td>Rapamycin</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>RU</td>
<td>RU486 (GR antagonist)</td>
</tr>
<tr>
<td>S6</td>
<td>Ribosomal Protein S6</td>
</tr>
<tr>
<td>s.c.</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>SCI</td>
<td>Spinal Cord Injury</td>
</tr>
<tr>
<td>SNI</td>
<td>Spared Nerve Injury</td>
</tr>
<tr>
<td>SNL</td>
<td>Spinal Nerve Ligation</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal Transducers and Activator of Transcription</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming Growth Factor</td>
</tr>
<tr>
<td>TENS</td>
<td>Transcutaneous Electrical Nerve Stimulation</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor Necrosis Factor Alpha</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like Receptor</td>
</tr>
<tr>
<td>UTP</td>
<td>Uridine Triphosphate</td>
</tr>
<tr>
<td>Veh</td>
<td>Vehicle</td>
</tr>
<tr>
<td>VIP</td>
<td>Vasointestinal Peptide</td>
</tr>
</tbody>
</table>
Chapter 1: Introduction

Introduction to Pain Terminology

Pain is an evolutionarily conserved and highly adaptive sense. It alerts the body of noxious stimuli to prevent or limit tissue damage. Of similar importance is the resolution of pain with healing. Indeed, chronic pain is pathological and represents a significant societal problem. Pain is a sensation that is distinguished from its sensory relative, touch, by negative sentiment; accordingly, the definition of pain includes a subjective perceptual component: “An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage” (Merskey and Bogduk, 1994). Notice also that the definition permits the possibility that tissue damage may not be detected.

Of pain’s relation to other senses, it has been said: “It has more in common with the phenomena of hunger and thirst than it has with seeing or hearing” (Wall et al., 1979). To classify pain and guide treatment, pain is characterized according to anatomical location, modality, intensity, quality and chronicity. Pain in specific dermatomes or bodily systems may indicate its source. Modality describes sensitivity to particular stimuli, such as heat, cold, touch, pin-prick and pressure, and often whether the pain-generating stimulus is dynamic (moving) or punctate (still). Pain intensity is subjective, but often is corroborated by physical signs including sympathetic indicators of sweat,
heart rate, blood pressure, and appropriate emotionality. **Quality** is a pain descriptor, for example if the pain feels like burning, shooting, stabbing, aching, cold, etc. A painful response to a typically non-noxious stimulus (e.g., touch, warmth) is called ‘allodynia.’ For acute pain, the most common example of allodynia is sensitivity to touch after sunburn. ‘Hyperalgesia’ describes increased pain to a noxious stimulus. Using the same analogy for hyperalgesia, exposure of sunburned skin to hot water becomes especially painful. ‘Dysesthesia’ can be used to describe either allodynia or hyperalgesia, as it refers more generally to unpleasant, abnormal sensations (e.g., burning, tingling); whereas ‘paresthesia’ describes an abnormal sensation not reported as painful (e.g., tingling, numbness, pins and needles). A selection of technical terms is summarized in Table 1.

<table>
<thead>
<tr>
<th>Pain Terminology</th>
<th>Definitions*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pain</td>
<td>An unpleasant sensory and emotional experience associated with actual or potential tissue damage</td>
</tr>
<tr>
<td>Neuropathic Pain</td>
<td>Pain initiated or caused by a primary lesion or dysfunction in the nervous system</td>
</tr>
<tr>
<td>Allodynia</td>
<td>Pain due to a stimulus which does not normally provoke pain</td>
</tr>
<tr>
<td>Hyperalgesia</td>
<td>An increased response to a stimulus which is normally painful</td>
</tr>
<tr>
<td>Dysesthesia</td>
<td>An unpleasant abnormal sensation, whether spontaneous or evoked</td>
</tr>
<tr>
<td>Paresthesia</td>
<td>An abnormal sensation, whether spontaneous or evoked</td>
</tr>
<tr>
<td>Hypoalgesia</td>
<td>Diminished pain in response to a normally painful stimulus</td>
</tr>
<tr>
<td>Nociceptor</td>
<td>A receptor preferentially sensitive to a noxious stimulus</td>
</tr>
<tr>
<td>Noxious Stimulus</td>
<td>A noxious stimulus is one which is damaging to normal tissues</td>
</tr>
</tbody>
</table>

*This definition has been reproduced with permission of the International Association for the Study of Pain® (IASP®). The definition may not be reproduced for any purpose without permission.

Table 1. Pain Terminology
Acute vs. Chronic Pain

Acute pain is not pathological; it accompanies tissue injury and resolves with proper healing. During recovery, pain motivates protection of the affected area against further damage. Chronicity is another element of pain classification because pain persistence beyond healing suggests a pathological process. Indeed, it has been suggested that acute should be replaced with ‘physiological’ or ‘normal;’ whereas, chronic should be replaced with ‘pathological’ in descriptions of chronicity (Kumazawa, 2004). In establishing whether pain is ‘chronic,’ it may be difficult to determine the extent of healing, thus it is common in clinical practice to apply fixed time durations. In back pain, for instance, pain lasting longer than six months is considered chronic. Such durations are relatively arbitrary and differ among conditions; in general, it is unknown if duration is related to mechanisms of chronic pain. Approximately 5-20% of cases of acute pain lead to chronic pain (Jensen, 2005; Jung et al., 2004; Kehlet et al., 2006).

Chronic Pain

Chronic pain is a major world health problem. When pain outlasts expected healing of the precipitating cause or injury, it is considered ‘chronic.’ According to the World Health Organization, an estimated 20% of the world population suffers from chronic pain. Pain is the most (or second most) common reason to seek physician consultation in the United States and a leading cause of disability (Turk and Dworkin, 2004). Still, it remains an under-recognized and under-treated medical problem. Quality of life is severely diminished by persistent pain; it imposes limitations on physical activity, from
exercise to walking and driving, and sexual activity. Such restrictions often impact the ability to maintain a typical lifestyle or job. There is also a significant psychological burden associated with chronic pain, either related to the lifestyle limitations or perhaps as a consequence of the pathophysiology itself. Not surprisingly then, are the numerous comorbidities of chronic pain, including stress, depression, anxiety and sleep disturbance. In general, social support improves health, but an unfortunate common consequence of this affliction is isolation and the dissolution of interpersonal relationships. The economic costs of chronic pain further justify increased attention to pain research. The cost of chronic pain to the U.S. is estimated at $100 billion annually (Stewart et al., 2003).

Chronic pain is a disease with pathological side effects (Tracey and Bushnell, 2009). In recent years, scientists have begun to identify long-term consequences of living with pain. It is clear from such efforts that chronic pain sufferers lose brain mass and show altered brain region connectivity and function (Cauda et al., 2009; Geha et al., 2008; Malinen et al., 2010). Across persistent pain conditions, neocortical regions exhibit atrophy, whereas other regions (e.g., hippocampus, thalamus) show some condition specificity (Apkarian et al., 2004a; Kuchinad et al., 2007; Schmidt-Wilcke et al., 2006). In line with these observations, chronic pain produces cognitive deficits. Emotional decision-making, attention and memory are impaired in individuals with chronic pain (Apkarian et al., 2004a; Dick and Rashiq, 2007; Sjogren et al., 2005). Despite cognitive impairment and brain atrophy, there are perhaps contrasting signs of plasticity in chronic
pain, including cortical reorganization and increases in gray matter volume (Vartiainen et al., 2009; Younger et al., 2010). Such plasticity is difficult to interpret, but may be associated with pain persistence. For example, individuals with chronic back pain show elevated resting activity of the medial prefrontal cortex with a concomitant inability to deactivate this area during a task (Baliki et al., 2008). Other physiological effects are under investigation, and an alteration of endocrine and immune function by chronic pain is likely (Anderson et al., 2009; Blackburn-Munro and Blackburn-Munro, 2003).

Neuropathic Pain

Introduction

Perhaps the most devastating type of persistent pain is neuropathic (Smith et al., 2007; Torrance et al., 2006). The International Association for the Study of Pain defines neuropathic pain as, “Pain arising as a direct consequence of a lesion or disease affecting the somatosensory system” (Treede et al., 2008). As such, diverse conditions and events precipitate neuropathic pain, including stroke, diabetes mellitus, herpes zoster infection, multiple sclerosis, nerve injury, amputation, cancer, and HIV; treatments for cancer and HIV also produce neuropathic pain (Bennett, 1998; Dworkin et al., 2003). The diversity in etiology of pain conditions can be reduced to fewer, broader classifications of traumatic, chemical, metabolic, and degenerative nervous system damage (Dworkin et al., 2003). For example, constricted blood flow to distal extremities in diabetes produces an injurious metabolic famine for nerves. Chemotherapeutic agents likely produce
chemical damage to nerves (Polomano et al., 2001; Xiao and Bennett, 2008). A more complete list of human neuropathic pain conditions is shown in Table 2.

<table>
<thead>
<tr>
<th>Clinical Neuropathic Pain (NP) Conditions</th>
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</thead>
<tbody>
<tr>
<td><strong>Peripheral NP</strong></td>
</tr>
<tr>
<td>Diabetes</td>
</tr>
<tr>
<td>Herpes Zoster (Shingles)</td>
</tr>
<tr>
<td>Trigeminal Neuralgia</td>
</tr>
<tr>
<td>Traumatic Nerve Injury</td>
</tr>
<tr>
<td>Nerve Compression</td>
</tr>
<tr>
<td>Tumor Invasion</td>
</tr>
<tr>
<td>Cancer Chemotherapy</td>
</tr>
<tr>
<td>Spinal Radiculopathy</td>
</tr>
<tr>
<td>Carpal Tunnel Syndrome</td>
</tr>
<tr>
<td>Demyelinating Polyradiculopathy</td>
</tr>
<tr>
<td>HIV</td>
</tr>
<tr>
<td>Iatrogenic Neuralgias</td>
</tr>
<tr>
<td>Radiation</td>
</tr>
<tr>
<td><strong>Central NP</strong></td>
</tr>
<tr>
<td>Spinal Cord Injury</td>
</tr>
<tr>
<td>Spinal Stenosis</td>
</tr>
<tr>
<td>HIV</td>
</tr>
<tr>
<td>Multiple Sclerosis</td>
</tr>
<tr>
<td>Parkinson Disease</td>
</tr>
<tr>
<td>Phantom Limb</td>
</tr>
<tr>
<td>Ischemia/Stroke</td>
</tr>
<tr>
<td>Radiation</td>
</tr>
</tbody>
</table>

Table 2. Human Neuropathic Pain Conditions

Pain of this origin is often intractable, due in part to mechanisms of substantial nervous system plasticity and persistent inflammation, and associated with significant co-morbidities, poor quality of life, and loss of function (reviewed in (Scadding and Koltzenburg, 2006). An estimated four to five million Americans suffer from neuropathic pain of different etiologies (Bennett, 1998); with an aging population and
improvements in diagnostics, this estimate is expected to increase (Bennett, 1998; Raja and Haythornthwaite, 2005).

**Diagnosis**

Neuropathic pain develops in only a proportion of patients with associated conditions. Specifying the presence of neuropathic pain, versus musculoskeletal or inflammatory pain is difficult. Not only does the diagnosis direct pain management strategies, it is pertinent to cost; insurance coverage for interventional treatment and pharmacotherapy relies on such diagnoses. Except in cases of clear neurological involvement (e.g., stroke, spinal cord injury, peripheral nerve trauma), or well-described neuropathic etiology (e.g., herpes zoster), classification of symptoms as neuropathic pain is challenging. Most low back pain, for instance, is of mixed etiology. There are often musculoskeletal and neuropathic components in back, and other joint pains. To distinguish neuropathic pain with some certainty requires extensive testing, and is referred to as quantitative sensory testing. According to the definition, first, a patient history should reveal probable neurologic disease or condition, and the clinical examination should reveal positive and/or negative sensory signs. Further diagnostic tests help to solidify the presence of a neuropathic condition. Based on these, a grading system is applied that dubs an individual as having possible, probable, or definite neuropathic pain (Cruccu et al., 2010).
History

Pain related to neural injury has been acknowledged for millennia. Contemporary pain vocabulary derives from ancient Greek texts, including 8th century B.C. in Homer’s writings and later, in 3rd century B.C. from the Hippocratic Collection (Rey, 1995). Although rudimentary, theories on the mechanisms of pain from Antiquity to the 19th century were not untenable. Before the popularization of ‘neuropathic pain’ as a descriptor, both ‘neuralgia’ and ‘causalgia’ described pain of this etiology. François Chaussier is credited with defining neuralgia in an 1802 publication (Rey, 1995). With a focus on trigeminal neuralgia, he describes the temptation of suicide as a consequence of such pain:

They [pains] are so sharp that patients willingly submit to the most painful operations without flinching and they themselves ask for ...the cruelest cauterizations; in the hope of putting an end to their ills, they desperately wish for the succor of the scalpel which is generally so dreaded. ...and no longer believing in either the power of medical craft or of nature, have finally put an end to a miserable existence which had become the most awful of tortures for them (Ibid, p 512).

Later, the painful syndrome ‘causalgia’ was described in 1872 by S. Weir Mitchell. Mitchell was a neurosurgeon during the American Civil War era. In his book, Injuries of Nerves and their Consequences, Mitchell provides notably insightful descriptions of underlying pathology associated with nerve injury. Of pain endured by soldiers with nerve damage, he says:
Under such torments the temper changes, the most amiable grow irritable, the soldier becomes a coward, and the strongest man is scarcely less nervous than the most hysterical girl (p 196; 1872).

Here, he captures the psychological aspects associated with neuropathic pain that still plague sufferers and gain research attention today. Furthermore, Mitchell documented distinctions between peripheral versus central pain, and reported the commonly described burning sensation after nerve damage (http://www.painonline.org/mitchell.htm). The term ‘causalgia’ was later replaced by ‘reflex dystrophy syndrome’ (RSD) and then ‘complex regional syndrome type II’ (CRPS II). In the early 1900s, French physicians, Dejerine and Roussy describe present-day neuropathic pain that derives from thalamic ischemia, called ‘thalamic syndrome’ (Pearce, 1988). Fairly modern versions of surgical procedures (e.g., cordotomy, sympathectomy, rhizotomy) to eradicate chronic nerve pain were developed the early to mid-1900s by, among others, Spiller, Frazier, Rose, Abbe, and Leriche (Rey, 1995). It is difficult to pin-down the first use of the term ‘neuropathic pain,’ but its use in publications shows up in the late 1970s (Dyck et al., 1976; Turnbull et al., 1980). Definitions of the similar conditions, neuralgia, causalgia and central pain were established in 1979 and endorsed by IASP (Merskey, 1979). A definition for neuropathic pain was established in 1994 by IASP (Merskey and Bogduk, 1994) and has since been revised (Treede et al., 2008).
Clinical Management: Pharmacotherapy

Presumably related to the complexity and heterogeneity of conditions, neuropathic pain is very difficult to manage. In the community and in randomized clinical pharmacotherapy trials, fewer than half of individuals experience pain relief, and for most, it is only partial (Dworkin et al., 2010; O’Connor and Dworkin, 2009). Coupled with adverse side effects, subpar efficacy makes many drugs undesirable to pain sufferers. Most drug trials are directed at pain populations of post-herpetic neuralgia and peripheral diabetic neuropathy, therefore the ability to extrapolate efficacy to other conditions is unknown (Dworkin et al., 2010). Recently the IASP Neuropathic Pain special interest group (NeuPSIG) established guidelines for the treatment of neuropathic pain (Dworkin et al., 2010). The authors propose a stepwise strategy that combines recommended first- and second-line medications with other therapies.

Based on clinical trial measures of efficacy, first-line medications include tri-cyclic antidepressants with serotonin and norepinephrine reuptake inhibition, such as duloxetine, venlafaxine and nortriptyline. Besides demonstrating efficacy in non-depressed individuals with neuropathic pain, depression is a common comorbid factor that may also be improved. Another first-line choice is anticonvulsants. Specifically, the calcium channel alpha2-delta ligands, gabapentin and pregabalin, are recommended. The final first-line recommendation is the topical lidocaine 5% patch (Dworkin et al., 2010). Lidocaine is a sodium channel inhibitor and its efficacy is believed to be due to local reduction of neural transmission.
Opioids comprise the second-line medication recommendation (Dworkin et al., 2010). Tramadol, specifically, is suggested as it has weak mu-opioid receptor activity as well as serotonin and norepinephrine reuptake inhibitor mechanisms. The risk of abuse, as well as a range of adverse side effects, generally limits the prescription of opioids by physicians.

The above recommended medications have been used for neuropathic pain for decades. What medications are on the horizon? The NeuPSIG guidelines (Dworkin et al., 2010) highlight trials for botulinum toxin A (Ranoux et al., 2008), as well as a high-concentration topical capsaicin patch (Backonja et al., 2008; Simpson et al., 2008). The recognition that neuropathic pain is heterogeneous within and across conditions has stimulated a mechanisms-based approach to identifying optimal pharmacotherapy for individuals (Turk, 1990; Woolf, 2004b). By determining patterns in symptoms that correspond to underlying pathology, pain scientists and physicians may one day be capable of designating mechanism-specific pharmacotherapy (Dworkin et al., 2010).

*Clinical Management: Non-pharmacological*

Surgical intervention is only useful for a subset of neuropathic pain conditions. Ablation procedures are rare and controversial in present-day medicine but, in the past, included dorsal rhizotomy, spinthalamic tract resection, and sympathectomy (Abbe, 1896; Meyerson, 1979; Nashold and Ostdahl, 1979; Rey, 1995; Spiller and Martin, 1912).
Long-term efficacy of these procedures was limited, perhaps due to progression of the underlying pathology, or nervous system plasticity that permits reorganization and/or regeneration of affected pathways. Modern therapy typically includes neurostimulation techniques, including transcutaneous and deep tissue stimulation. Transcutaneous electrical nerve stimulation (TENS) is the least invasive method, employing high and low frequency to activate large diameter Aβ fibers. The efficacy is short-lived and thus requires frequent daily use (Cruccu et al., 2007). Spinal cord stimulators can be implanted into the epidural space at the affected level and controlled with remote activators. These show improved efficacy compared to TENS, but obviously are invasive and thus more risky (Cruccu et al., 2007). Deep brain stimulation of the thalamus and periventricular grey matter is similarly efficacious and more invasive. Finally, motor cortex stimulation (MCS) is a more recent development for neuropathic pain management (Garcia-Larrea and Peyron, 2007). It offers long-duration efficacy of hours to days, and like deep brain stimulation, is believed to work by activating inhibitory descending pathways. Additionally, MCS may activate orbitofrontal-cingulate cortex to quell emotional aspects of pain (Marchand et al., 2009). Nerve root compression that typically presents as radiating limb pain and other neuropathies can transiently improve with epidural injections of anesthetic and steroid (Buenaventura et al., 2009; van Wijck et al., 2006). Finally, among the least invasive non-pharmacological treatment strategies is physical therapy. Muscle strengthening exercises affect posture and circulation (among other factors) to alleviate pain.
Experimental Models of Neuropathic Pain

Rodent models of neuropathic pain most commonly utilize peripheral nerve injury procedures to produce clinically relevant behavioral and physiological endpoints, and to test efficacy of prospective pain-relieving medications (Ossipov et al., 2006). A frequently posed question to pain scientists is how pain can be measured in non-human animals. As stated above, pain is a subjective experience and would seemingly require lingual confirmation. Numerous methods have been developed, however, to identify and measure behaviors that suggest pain communication in non-human animals. In rodents and other species, these measures can be divided into spontaneous and evoked behaviors. Spontaneous pain, or pain without an apparent stimulus, is a primary complaint in human neuropathic pain. After peripheral nerve injury, we observe spontaneous behavior in rodents including lifting and guarding of the hind paw. Other examples of behavior that imply pain include postural changes that minimize weight bearing, reduction in typical locomotive behavior, and analgesic self-administration. It is important to note, however, that abnormal paw posturing could represent motor effects. Loss of typical sleep or food intake may indicate spontaneous pain, and is certainly a correlate of pain in humans. However, we recognize that such behaviors may represent an indirect outcome of experimentally-induced physiological changes. For face validity and thus translational potential, spontaneous measures are preferred; unfortunately, the specificity of these behaviors is imprecise and quantitation of these behaviors requires tedious, long-duration recording and/or costly equipment to capture instances of behavior. For this reason, most behavioral studies report measures of evoked pain-like behaviors. Operant conditioning,
by some, is considered a measure of spontaneous behavior (Vierck et al., 2002; Vierck et al., 2004). Operant tests introduce a motivational component that is inextricable from an analgesic effect (Campbell and Meyer, 2006), and stimulus-evoked testing has an inherent motor component that cannot be dissociated from the sensory component. For review of this topic, see (Charles et al., 2005; Jeffrey and Sara, 2005). In summary, both approaches to understanding pain-like behavior in rodents are imperfect; still, the predictability of translating pharmacotherapy from rodents to humans is generally quite good using commonly employed stimulus-evoked assays (LaBuda and Little, 2005).

What are stimulus-evoked behaviors that imply pain? To assess pain-like behavior in rodents, a baseline (normal) threshold of hind paw sensitivity can be acquired for cold, heat, or tactile stimulation. The threshold at which the animal withdraws its hind paw is interpreted as an uncomfortable, or painful, stimulus. Then, thresholds are assessed after a pain-evoking condition is established (e.g., after nerve injury or inflammation). Lowered thresholds to the previously tested stimulus are interpreted as pain-like behavior. The validity of this assumption is confirmed by pharmacological reversal of hind paw sensitivity after the development of such conditions. Tactile mechanical sensitivity is determined using nylon monofilaments that produce calibrated forces when bent on the tissue surface; these are called von Frey hairs after Max von Frey, who in the 1890s created the test and correlated sensation with nerve ending structure (Perl, 2007). Sensitivity to normally innocuous hairs is interpreted as allodynia-like behavior. Sensitivity to heat can be tested by measuring paw withdrawal latency on a hot surface.
(~55°C). The hot plate exposes both hind paws to the stimulus; a unilateral test can be accomplished using the Hargreaves’ test wherein an infrared beam is projected on the hind paw. A photocell attached to a timer indicates the duration until hind paw withdrawal. Because the heat stimulus is normally ‘painful,’ shorter latencies are interpreted as ‘hyperalgesia.’ Numerous other tests are suitable for testing hind paw sensitivity to specific modalities. A list of common tests is provided in Table 3.

<table>
<thead>
<tr>
<th>Measures of Spontaneous Pain</th>
<th>Measures of Evoked Pain</th>
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</thead>
<tbody>
<tr>
<td>Posture/Weight Bearing</td>
<td>Allodynia</td>
</tr>
<tr>
<td>Gait</td>
<td>Tactile (von Frey Test)</td>
</tr>
<tr>
<td>Exploratory Behavior/Locomotion</td>
<td>Cold Plate</td>
</tr>
<tr>
<td>Rearing</td>
<td>Hyperalgesia</td>
</tr>
<tr>
<td>Climbing/Grip Strength</td>
<td>Hot Plate</td>
</tr>
<tr>
<td>Nocifensive Behavior</td>
<td>Hargreaves' Test</td>
</tr>
<tr>
<td>Paw licking</td>
<td>Randall-Selitto Paw Pressure</td>
</tr>
<tr>
<td>Overgrooming</td>
<td>Cold (Acetone)</td>
</tr>
<tr>
<td>Guarding</td>
<td></td>
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<tr>
<td>Autotomy</td>
<td></td>
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<tr>
<td>Feeding</td>
<td></td>
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<tr>
<td>Sleep</td>
<td></td>
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<tr>
<td>Affective Behavior</td>
<td></td>
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<tr>
<td>Analgesic Self-administration</td>
<td></td>
</tr>
<tr>
<td>Operant Conditioning</td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Rodent Measures of Pain-like Behavior

To study neuropathic pain in the laboratory, a variety of procedures that mimic human neuropathic pain are applied to rodents and other species. Sciatic nerve injuries are most
commonly utilized for several reasons: 1) the procedures are relatively simple and reproducible; 2) unlike systemic treatments, sciatic injury produces direct and easily examined damage; 3) the affected dermatomes include the hind paw, which is amenable to behavioral testing. One may wonder why there are so many models of peripheral nerve injury. Attempts to improve on specific aspects of the models have produced several variations. The first rodent model of neuropathic pain involved complete transection of the sciatic nerve (Wall et al., 1979). Self-injurious behavior, or autotomy, limited the use of this model. Subsequently, chronic constriction injury (CCI) was developed. It is produced by the placement of four loose chomic gut ligatures on the sciatic nerve (Bennett and Xie, 1988). The chomic gut suture is inflammatory, therefore the combined constriction of the ligatures with inflammatory edema produces damage and subsequent pain-like behaviors (e.g., spontaneous behaviors, thermal hyperalgesia, tactile and cold allodynia) lasting for approximately two months (Maves et al., 1993). This model is heralded for modeling pain of mixed etiology, for example the inflammatory and neuropathic components of back pain with radiculopathy (Campbell and Meyer, 2006). Its shortcomings include potential for imprecise reproducibility in ligature tightness and spacing, as well as the development of self-injurious behavior, or autotomy (Walker et al., 1999). The Seltzer model followed in 1990, which produces fast onset (hours) and long-lasting (~ 7 months) pain-like behaviors by ligating one-third to one-half of the sciatic nerve (Seltzer et al., 1990). In addition to ipsilateral hindpaw sensitivity, this model often produces mirror-image pain-like behaviors (i.e., pain in the contralateral hindpaw). Like the Bennett (CCI) model, the Seltzer model can pose issues
of reproducibility related to the tightness and extent of sciatic ligation. The Chung model of spinal nerve ligation was established to mimic nerve root compression; although it now has variations, the original model involved unilateral ligation of the L5 or the L5 and L6 spinal nerves (Kim and Chung, 1992). An advantage to the Chung model is that, by ligating at the level of the spinal nerve, the pathology is limited to discrete spinal cord segments, which is in contrast to sciatic nerve lesions that affect multiple segments. A disadvantage is the difficulty of the procedure that requires extensive bone and muscle damage; in a mouse (because of its small size relative to rat), this procedure poses threat of damage to the spinal cord cauda equina (Walker et al., 1999). Variations on injury to the branches of the sciatic nerve (common peroneal, tibial and sural nerves) have also been developed, and termed spared nerve injury (SNI) (Decosterd and Woolf, 2000; Shields et al., 2003). The advantage of selective branch injury is distinct innervation of the hind paw by individual branches, thus enabling medial versus lateral paw testing. One disagreement among the models is the development of thermal hyperalgesia. For unknown reasons, thermal sensitivity is not present or robust in all models, including SNI, PSNL (partial sciatic nerve ligation), and sciatic transection (Dowdall et al., 2005). Most of the above described models were developed in rat but have also been adapted for use in mice (Malmberg and Basbaum, 1998; Ramer et al., 1998). A list of common experimental models of neuropathic pain is provided in Table 4.
Common Experimental Models of Neuropathic Pain

<table>
<thead>
<tr>
<th>Model</th>
<th>Description</th>
<th>Illustration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic Constriction Injury 1 (CCI)</td>
<td>Four loose ligatures on sciatic nerve</td>
<td></td>
</tr>
<tr>
<td>Spinal Nerve Ligation 2 (SNL)</td>
<td>Ligation and/or cut of L5 and/or L6 spinal nerve</td>
<td></td>
</tr>
<tr>
<td>Partial Sciatic Nerve Ligation 3 (PSNL)</td>
<td>Ligation of 1/3 to 1/2 of sciatic nerve</td>
<td></td>
</tr>
<tr>
<td>Spared Nerve Injury (SNI)</td>
<td>Ligation, transection and resection of two sciatic branches</td>
<td></td>
</tr>
<tr>
<td>Sciatic Crush Injury</td>
<td>Crush trauma to sciatic nerve for varying durations</td>
<td></td>
</tr>
<tr>
<td>Spinal Cord Injury</td>
<td>Various models of injury to spinal cord</td>
<td></td>
</tr>
<tr>
<td>Diabetic Neuropathy</td>
<td>Transgenic diabetic mice or systemic streptozotocin</td>
<td></td>
</tr>
<tr>
<td>Chemotherapeutic Agents</td>
<td>Systemic delivery of vincristine or paclitaxel</td>
<td></td>
</tr>
</tbody>
</table>

1Bennett & Xie, 1988; 2Kim & Chung, 1992; 3Seltzer et al., 1990

Table 4. Rodent Models of Neuropathic Pain

Peripheral nerve injuries predominate this field of research; however, models that mimic diverse neuropathic pain states in human have also been developed. Rodent spinal cord injury (Detloff et al., 2008; Hulsebosch et al., 2009; Yezierski, 2000), cancer (Schwei et al., 1999), cancer chemotherapy (Chaudhry et al., 1994; Smith et al., 2004), diabetes (Courteix et al., 1993), stroke and ischemic-hypoxic injury (unpublished data) are utilized. The underlying mechanisms for neuropathic pain of central origin, as in stroke or spinal cord injury may vary considerably from peripheral nerve injury (and from each other). Few studies have investigated changes in the brain due to peripheral nerve or
spinal cord lesion related to pain (Zhao et al., 2007). Likewise, examples of peripheral nerve studies are limited after spinal cord or brain injury.

For numerous reasons, the feasibility of testing human neuropathic pain mechanisms is poor. How well, then, do experimental models represent human pathology? For neuropathic pain indications, pre-clinical drug testing is often accomplished using peripheral nerve lesions (Iyengar et al., 2004; Jarvis et al., 2002; Sullivan et al., 2007; Valenzano et al., 2005). For four out of five commonly prescribed medications for neuropathic pain, rodent nerve lesion studies predict efficacy and adverse events (Whiteside et al., 2008). There is one definitive failure of rodent-to-human translation. Antagonists of the neurokinin-1 (NK-1) receptor, of which substance P is the ligand, were successful in attenuating pain-like behavior in rodents (Hill, 2000; Rice and Hill, 2006). However, when tested clinically, there was no benefit (Boyce and Hill, 2000). Another neuropeptide target appears to be promising, however; CGRP inhibition is efficacious in experimental models and in humans (Ballet et al., 2001; Shembalkar et al., 2001). Cholecystokinin (CCK) inhibitors have also demonstrated potential as adjuncts to morphine. CCK inhibitors enhance morphine analgesia in humans (McCleane, 1998). In addition to pharmacological translation, the rodent neuropathic pain models produce behavioral hypersensitivities, (e.g., allodynia, hyperalgesia) similar to human pain qualities. Overall, advancements in basic pain research appear to be useful for human application. Disparities in mechanism, or spontaneous pain versus hypersensitivity
outcomes between humans and rodents should be considered, however, when pharmacotherapy does not translate successfully from rodent studies.

**Mechanisms of Neuropathic Pain**

*Overview of Sensory Transmission Anatomy*

Sensing pain requires the processes of transduction, conduction, transmission, and perception (Woolf, 2004b). Noxious stimuli are ‘transduced’ into electrical activity at the peripheral terminals of nociceptors. The transducers are often chemical, thermal, or mechanical sensing ion channels. If this electrical activity generates action potentials, then the propagation of the action potential along axons to the CNS is ‘conduction.’ ‘Transmission’ is the synaptic transfer of input along anatomical circuitry. The brain is among the destinations of nociceptive transmission, and ‘perception’ is the cognitive component determined by integration and processing of signals both affective and somatosensory. Below is a brief description of the anatomy involved in pain perception.

Axons that convey sensory information (e.g., touch, proprioception, pain) are bundled together with motor axons in nerves. Individual motor and sensory axons terminate in specialized tissues to perform specific functions (e.g., motor axons innervate muscle fibers, whereas sensory axons innervate skin, joints, muscles, connective tissue, etc.). Schwann cells myelinate sensory axons that convey proprioceptive, tactile, and vibratory sensation; myelinated axons exhibit increased diameter and conduction velocity. An axon with its constituents (e.g., myelin or ensheathing glial cells) is termed a ‘nerve
fiber;’ fibers are classified by function, diameter, and conduction speed. These classifications for myelinated fibers are termed: ‘A/I’ (with alpha, beta, and delta (III) subclasses), ‘B,’ or ‘II,’ where each class has distinct properties. Unmyelinated fibers are termed ‘C’ (or ‘IV’) fibers; lacking myelin, C fibers exhibit the smallest diameter and slowest conduction velocity. Unmyelinated and lightly myelinated (A-delta) sensory axons convey pain and temperature sensations. Near the spinal cord, motor and sensory axons split into distinct nerves that form the ventral (motor) and dorsal (sensory) roots.

The cell soma of peripheral sensory axons cluster in the dorsal root ganglia (DRG). The DRG is located along the dorsal aspect of the spinal nerve, and the nerve segment between the DRG and spinal cord is called the dorsal root. Each spinal cord level has respective left and right side DRGs. DRG neurons lack dendrites; they are pseudo-unipolar cells with peripheral and central projecting axon processes. The peripheral sensory axon is referred to as a primary afferent because it forms the first projection of sensory input to the CNS. In summary, DRG neurons are composed of an axon that projects peripherally to target organs and centrally to the spinal cord dorsal horn.

The dorsal horn is the site of the first sensory synapse in the spinal cord. Here, the central axon of the DRG neuron (first-order neuron) synapses onto CNS neurons, termed second-order neurons. The phenotype of the axon determines its course in the CNS. Large diameter, myelinated A fibers enter the spinal cord dorsal horn and bifurcate; one branch synapses in deep dorsal horn to mediate motor reflexes, the other branch ascends
through the dorsal columns to synapse on second-order neurons in the gracilis or cuneatus nuclei in the medulla. In those nuclei, the second-order neuron decussates and ascends to the thalamus, synapsing on third-order neurons that project to sensory cortex. This pathway is referred to as the dorsal column-medial lemniscus system and, in general, its input provides proprioceptive and crude touch information.

The small diameter, unmyelinated C fibers and lightly myelinated A-delta fibers form the anterolateral, or spinothalamic, ascending sensory pathway. First-order afferents enter the dorsal horn in the dorsolateral tract of Lissauer. Synapses onto second-order neurons can occur in several of Rexes’ laminae, including I, IV, V, VI, VII and VIII (Hodge and Apkarian, 1990; Noback et al., 1991; Willis and Coggeshall, 1991; Young, 1986). Second-order neurons decussate in the ventral white commissure of the spinal cord, and synapse on third-order neurons in the thalamus that project throughout the brain including cortex and limbic areas. Widespread innervation of the anterolateral tract yields both discriminative and emotional qualities of pain and temperature information.

Persistent pain may result from pathology at any of these levels of sensory transmission. In most experimental models of neuropathic pain, the peripheral nerve is injured; however, the pathology is not limited to the site of damage. Aberrant signals are propagated along the anatomical transmission pathways and may manifest as pathology that is maintained independent of the original injury. The mechanisms that underlie the
development and maintenance of neuropathic pain are described in the next section.
Table 5 provides a list of cellular and molecular mediators of neuropathic pain.

Mechanisms: Neuron Plasticity

Ectopic Excitability

After sensory nerve injury, the injured neurons or neighboring neurons can develop ectopic, or stimulus-independent, activity (Blumberg and Janig, 1984; Liu et al., 2000a; Seltzer and Devor, 1979). This spontaneous action potential firing may originate at the site of injury or in the DRG; most often it is observed in A fibers but can occur in C fibers (Devor and Seltzer, 1999; Grossmann et al., 2009). Molecular mechanisms implicated in ectopic firing include upregulation of voltage-gated sodium channels (subunits 1.3 and 1.8), and down regulation of potassium channels in injured neurons (Devor, 1983; Gold et al., 2003; Kim et al., 2002; Pertin et al., 2005; Roza and Lopez-Garcia, 2008). In the dorsal horn, peripheral ectopic activity may trigger increased excitability of second order neurons to produce central sensitization, as described below. In effect, this aberrant peripheral neuron function is felt as pain or paresthesia despite the absence of a painful stimulus.

Peripheral Sensitization

The microenvironment of an injured axon is replete with factors that activate and/or sensitize peripheral nerve terminals. Among them are ATP, cytokines, chemokines, growth factors, and protons (that reduce pH). Peripheral sensitization is a state of
reduced activation threshold and increased responsiveness of the axon terminals that occurs in inflammatory and neuropathic pain (Djouhri et al., 2006; Woolf and Ma, 2007). As examples, ATP directly activates nociceptors by binding purinergic receptors (Burnstock, 2000) and acidic pH activates TRPV1 and ASIC channels (Holzer, 2009). NGF and PGE2, on the other hand, sensitize nociceptors without direct stimulation (Shu and Mendell, 1999). Bradykinin can both activate nociceptors and sensitize them to subsequent stimulation (Cesare and McNaughton, 1996; Ferreira et al., 2004; Oh and Weinreich, 2004; Thayer et al., 1988). Activation of the nociceptors by inflammatory mediators is another source of ectopic excitability as described above (Michaelis et al., 1998). Peripheral nerve fibers communicate neural damage to DRGs by both chemical axonal transport and electrophysiological signals (Delcroix et al., 2003; Schafers and Sommer, 2007). Rapid action potential barrage after peripheral nerve damage informs the DRG of the insult and this signal is propagated to the dorsal horn. Blocking peripheral sensitization and ectopic excitability with anesthetic (e.g., bupivicaine) reduces neuron discharge that prevents (but does not reverse) central sensitization and pain behavior (Suter et al., 2009; Wen et al., 2007).

Central Sensitization

Neural mechanisms of pain are often compared to long term potentiation (LTP) (Ji et al., 2003). LTP is best described for its role in hippocampal learning and memory, but broadly refers to activity-dependent enhancement of synaptic connections. For pain, the analogous term is ‘central sensitization’ (Woolf, 1983). The manifestation of central
sensitization after nervous system damage is a prominent mechanism of persistent pain (Woolf and Salter, 2000). Like peripheral sensitization, central sensitization describes reduced activation thresholds and increased responsiveness of dorsal horn neurons (or higher-level synapses). This amplification of synaptic input results initially from activity-dependent stimulation by the primary afferent (Woolf, 1983). Subsequently, a stimulus-independent, transcription-dependent mechanism sustains sensitization (Jin et al., 2003; Neumann et al., 1996; Woolf, 2004a). The net result is hyperresponsivity to innocuous and noxious stimulation (e.g., allodynia and hyperalgesia) (Campbell et al., 1988; Koltzenburg et al., 1992). Like LTP, this process is NMDAR dependent (Brenner et al., 2004; Kawasaki et al., 2004; Ma and Woolf, 1995; Woolf and Thompson, 1991) and transcription-dependent with significant contribution by pro-inflammatory gene expression (Kohno et al., 2008b; Samad et al., 2001; Wang et al., 2005a).

**Structural plasticity**

Peripheral and central sensitization reflects substantial neural plasticity. Neuroplasticity is also reflected in axon sprouting. Injury-induced sprouting of peripheral and central neurites is associated with the development of neuropathic pain. After nerve trauma, peripherally-projecting axons form neuromas that correlate with pain in humans (Gilmer-Hill et al., 2002), and exhibit ectopic firing potential (Fried and Devor, 1988). Centrally-projecting axon sprouts demonstrate nociceptor phenotypes (i.e., co-localization of GAP-43 with CGRP-labeled afferents) (Hu et al., 2004; Ondarza et al., 2003). Uninjured axons also exhibit plasticity; both small- and large-diameter axons spared from injury sprout
into the terminal fields of injured axons (Hu et al., 2004; Woolf et al., 1992; Woolf et al., 1995).

Synaptic plasticity may couple with structural plasticity (e.g., axon sprouting) to produce pain hypersensitivity. Hippocampal LTP activates a marker of axon sprouting, GAP-43, in an NMDA receptor-dependent manner (Namgung et al., 1997; Ramakers et al., 1999). A role for structural remodeling in functional neuroplasticity is supported by evidence that PKC phosphorylation of GAP-43 regulates memory processes (Holahan and Routtenberg, 2008) and LTP (Hulo et al., 2002). Together, these data suggest that LTP may facilitate axon growth, and conversely, mechanisms of axon growth overlap with synaptic plasticity. With regards to pain, few studies have tested the role of known growth proteins; however, GAP-43 expression in human pancreatic nerves correlates with pain in pancreatitis (Di Sebastiano et al., 1997). A common feature of both axon sprouting and synaptic plasticity is synaptogenesis (Tominaga-Yoshino et al., 2002). New synapse formation and reorganization after nervous system injury may facilitate neural transmission and the spread of hyperexcitability (Chou et al., 2002; Csillik and Knyihar-Csillik, 1981; Tominaga-Yoshino et al., 2002). Bidirectional and dual regulation of synaptic and structural plasticity after injury may exacerbate the pathogenesis of neuropathic pain.
Mechanisms of Neuropathic Pain

Immune Mechanisms

Inflammation, historically, refers to the presence of five cardinal clinical signs: tissue swelling, redness, heat, pain and loss of normal function. The purpose of an inflammatory response is to promote survival against injurious stimuli (e.g., pathogens, trauma). At a cellular and molecular level, inflammation is highly complex and its intricacies are beyond the scope of this discussion (Medzhitov, 2008) for review). Broadly, inflammation encompasses processes of plasma protein exudation and leukocyte extravasation, and such functions as vasodilation, coagulation, phagocytosis, release of proteases, chemokines and cytokines.

Neuroinflammation

When inflammation occurs in a neural tissue, it is often specified as ‘neuroinflammation.’ The healthy nervous system is not endowed with cells to support inflammation. The brain, spinal cord, and peripheral nerve, together with endothelia, must elicit peripherally-derived leukocytes to generate classical inflammation (Cui et al., 2000). Thus, ‘neuroinflammation’ describes induction of non-resident immune cell activity in nervous system tissues (Popovich and Longbrake, 2008). Microglia are resident cells of the CNS that, when activated, share functions with peripheral macrophages, and likely collaborate with peripherally-derived immune cells in neuroinflammation. For example, microglia can release pro-inflammatory cytokines, prostaglandins, proteolytic enzymes,
and nitric oxide. Like macrophages, microglia also can remove cellular debris by phagocytosis (reviewed in (Stoll and Jander, 1999; Streit et al., 1988; Streit et al., 1999).

In the past 20 years, neuroinflammation has gained recognition as a primary mechanism in the development of neuropathic pain in rodent peripheral nerve injury. Peripheral nerve injury (e.g., sciatic) triggers a spatio-temporally defined inflammatory response. The magnitude of the response is determined by the type of nerve damage; ligation injuries show increased measures of inflammation versus non-ligation (e.g., transection, crush) (Cui et al., 2000; Hu et al., 2007). This is intriguing as it suggests that inflammation is not proportional to the damage of endothelia, Schwann cells, and axons (that would be severed in transection), but rather, that the pathology is perhaps protracted and thus cumulative after ligation injuries. In support of this, Fry et al. demonstrated deficient macrophage efflux in ligated nerve versus crush (Fry et al., 2007). Finally, with respect to magnitude, the neuroinflammatory response is moderated with distance from the lesion site (Hu and McLachlan, 2003). Sites, time course, and roles of neuroinflammation after peripheral nerve damage are described below.

**Peripheral Nerve**

Common procedures of peripheral nerve damage disrupt the blood-nerve barrier or expose the nerve entirely (e.g., by transection). In neuropathic pain models, the lesion site typically harbors the most inflammation (Sorkin and Schafers, 2007). Here, Schwann cells, endothelia, and the few resident immune cells (e.g., mast cells) rapidly
respond to damage (Cui et al., 2000; Galli et al., 2005; Metcalfe et al., 1997; Zuo et al., 2003). Products of these early responders, including histamine, chemokines, pro-inflammatory cytokines (e.g., IL-1β, TNF-α), and proteases increase within an hour post-injury (Uceyler et al., 2007). These mediators excite neurons and aid in the initiation of inflammation via chemoattraction and vasodilation. Schwann cells, not classically considered immune cells, are myelinating resident nerve glia that transiently morph into pro-inflammatory, phagocytic cells (Bergsteinsdottir et al., 1991; Bolin et al., 1995; Chen et al., 2006; Reichert et al., 1994; Rutkowski et al., 1999; Shamash et al., 2002; Tofarids et al., 2002; Wagner and Myers, 1996). Neutrophils infiltrate as early as one hour and peak by 24 hours (Clatworthy et al., 1995; Perkins and Tracey, 2000; Perry et al., 1987), and preemptive neutrophil depletion minimizes pain-like behavior after injury (Perkins and Tracey, 2000). Hematogenous macrophages are recruited within 24 hours but peak later, approximately 7-28 days post-injury, and pain-like behavior temporally correlates with macrophage infiltration of the nerve (Myers et al., 1996). Neutrophils and macrophages release highly pro-inflammatory substances, including prostaglandins, lipoxygenases, cytokines, ROS, NO, proteases, and ATP. Importantly, virtually every agent produced by the described cells can exert direct or indirect stimulatory effects on neuron function (Sorkin and Schafers, 2007). As described in the previous section, neuron sensitization and activation after injury constitutes a principal pain mechanism. Exogenous administration of such agents (e.g., TNF-α, PGE2) results in pain-like behavior (Fukuoka et al., 1994; Jin and Gereau, 2006; Obreja et al., 2005; Obreja et al., 2002; Ozaktay et al., 2006; Schafers et al., 2003a; von Banchet et al., 2005; Zhang et al., 2002).
Immunosuppressive treatment (Bennett, 1999; Clatworthy et al., 1995) and macrophage deletion (Barclay et al., 2007; Liu et al., 2000b) reduce immune cell infiltration and pain-like behaviors. Of note, Rutkowski showed that macrophage deletion was without effect (Rutkowski et al., 2000). Encasing the nerve stump to reduce exposure of the nerve to inflammatory cells attenuates autotomy, a behavior indicative of sensory dysfunction (Okuda et al., 2006).

Nerve inflammation contributes substantially to pain behavior. The role of inflammation in peripheral axon regeneration is also worth consideration. Most available data indicate a pro-regenerative role of immune cells and their secretory products on regeneration (Barrette et al., 2008; Boivin et al., 2007; Horn et al., 2008; Lu and Richardson, 1991; Nishio et al., 2002; Rong et al., 2004; Yin et al., 2009; Zou et al., 2006). Yet, aberrant axon growth may lead to pain (Di Sebastiano et al., 1997; Fried and Devor, 1988; Gilmer-Hill et al., 2002; Woolf, 1983; Woolf and Salter, 2000), and measures of axon regeneration in vivo generally do not distinguish type of axon growth. It is plausible, then, that inflammation-supported regeneration could be pro-algesic. An example of this proposed phenomenon is the pro-inflammatory cytokine, macrophage migration inhibitory factor (MIF). As shown in Chapter 3, MIF is essential for neuropathic pain-like behaviors, and important also for axon regeneration (Nishio et al., 2002). Another example with similar outcomes is tissue plasminogen activator (tPA) (Hayden and Seeds, 1996; Siconolfi and Seeds, 2001; Yamanaka et al., 2004; Zou et al., 2006). Furthermore, the recent appreciation that inflammation, particularly by the macrophage, is not
homogeneous (Gensel et al., 2009; Gordon, 2003; Kigerl et al., 2009; Slobodov et al., 2001) suggests that specific aspects of nerve inflammation may facilitate productive regeneration while others produce detriment (e.g., aberrant growth and pain). Indeed, *in vitro*, classically activated, pro-inflammatory M1 macrophages support an axon-sprouting phenotype that has been implicated in pain, whereas alternatively activated, anti-inflammatory M2 macrophages support long-distance, non-branching growth indicative of regenerative growth (Kigerl et al., 2009). The specification of macrophage phenotype after peripheral nerve injury has not been evaluated. Future studies should phenotype peripheral nerve inflammation over time, as well as determine whether distinct inflammatory phenotypes direct pain, and sprouting versus long-distance axon regeneration.

*Dorsal Root Ganglion*

After injury to the peripheral nerve, immune cells also infiltrate the DRG. The resident glial population, satellite cells, hypertrophy, proliferate, and increase expression of numerous inflammatory and neuron support molecules (Ohtori et al., 2004; Takahashi et al., 2006; Woodham et al., 1989; Zhang et al., 2000; Zhou et al., 1999). Exposure of DRG neurons to pro-inflammatory cytokines excites nociceptive neurons and produces pain-like behaviors (Fukuoka et al., 1994; Ozaktay et al., 2006; Schafers et al., 2003b; von Banchet et al., 2005; Zelenka et al., 2005). After nerve injury, TNF-α and other cytokines are retrogradely transported to the DRG and subsequently to the spinal cord (Shubayev and Myers, 2001, 2002). Antidromic shuttling of TNF-α, and release into
target organs is also possible (Schafers et al., 2002), suggesting bi-directional pro-
inflammatory and neuro-excitatory communication. Activated kinases (e.g., MAPK),
likewise, can be anterogradely and/or retrogradely transported after nerve injury (Cavalli
et al., 2005; Delcroix et al., 2003; Perlson et al., 2005; Reynolds et al., 2001). Suppression of cytokine signaling or downstream intracellular kinase signaling is analgesic (Sommer et al., 2001a; Sommer et al., 2001b; Sweitzer et al., 2001b). Relative
to peripheral nerve and spinal cord, less attention has been directed at understanding
neuroimmune mechanisms of neuropathic pain in DRG.

Spinal Cord

Peripheral immune cell infiltration in spinal cord is considerably less than in the lesioned
nerve. In fact, only recent data implicate the presence of veritable ‘neuroinflammation.’
Sciatic nerve injury produces variable blood-spinal cord barrier permeability (Gordh et
al., 2006; Kwan et al., 2008) that can enable leukocyte extravasation. Indeed, T cells
enter the spinal cord after nerve injury and T cell deficient mice exhibit reduced pain
behaviors (Cao and DeLeo, 2008; Costigan et al., 2009; Sweitzer et al., 2002). Detection
of macrophage infiltration is obscured by indistinct microglial expression of common
markers (e.g., Iba-1, CD11b, MHC I & II). When CD45 expression is measured
coincident with CD11b, it is a useful tool to distinguish microglia (low CD45) from
macrophages (CD45 high) (Sedgwick et al., 1991). However, the relative expression of
CD45 on spinal cord CD11b+ cells has not been demonstrated after nerve injury.
Every model of nerve transection, ligation or crush injury produces dorsal horn microglial reactivity (DeLeo and Yezierski, 2001; Hashizume et al., 2000; Katsura et al., 2006; Meller et al., 1994; Raghavendra et al., 2003) that is characterized by cellular hypertrophy, migration, and/or proliferation (Streit et al., 1999; Tsuda et al., 2005; Tsuda et al., 2004). This shift from a surveying phenotype to a ‘reactive’ phenotype after injury is associated with increased expression of cell surface markers (e.g., CD11b, Iba-1, MHC-II, F4/80) and pro-inflammatory signaling molecules (e.g., cytokines, p-p38, ROS) (Hanisch and Kettenmann, 2007). The specificity of the microglial response is demonstrated by its spatially-restricted reactive zone. Essentially, microglia react in the zone of injured afferent terminals (Beggs and Salter, 2007). In some neuropathic models, microglial reactivity may be observed along the nociceptive pathway (e.g., PAG, thalamus, cortex) (Banati, 2002; Detloff et al., 2008; Zhao et al., 2007). The diffusion of this response may be dependent on injury severity or proximity to the spinal cord (Zhang et al., 2008).

Microglial reactivity appears to play a critical role in neuropathic pain. It is temporally associated with the development of pain behaviors (Watkins et al., 1995) and pre-injury treatment with the microglia inhibitor, minocycline, attenuates pain (Hains and Waxman, 2006; Hua et al., 2005; Ledeboer et al., 2005b; Narita et al., 2006b). Similarly, the mixed-glial inhibitors, fluorocitrate (Meller et al., 1994; Milligan et al., 2003; Watkins et al., 1997) and propentofylline (Garry et al., 2005; Sweitzer et al., 2001b) demonstrate efficacy in neuropathic pain. All three agents lack specificity for microglia; however,
other evidence implicating microglia in neuropathic pain is strong. For example, inhibition of microglial MAPK p38 and the ATP receptor, P2X₄ ameliorate pain-like behavior (Tsuda et al., 2005; Tsuda et al., 2003). Further, treatment with ATP-activated microglia produces neuropathic pain behaviors in the absence of nervous system injury (Coull et al., 2005; Narita et al., 2006a; Tsuda et al., 2003). Microglia may affect neuropathic conditions by releasing pro-algesic mediators that sensitize neighboring neurons. Following neural injury, the release of pro-inflammatory cytokines (e.g., TNF-α, IL-1, IL-6) and other pain mediators (e.g., prostaglandins, nitric oxide, reactive oxygen species, and glutamate) by activated microglia correlates with the development of behavioral sensitization, including hyperalgesia and allodynia (Coyle, 1998; DeLeo et al., 2004; Jin et al., 2003; Ledeboer et al., 2005a; Watkins et al., 2001).
**Mediators of Neuropathic Pain**

<table>
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<tr>
<th>Cells</th>
<th>Cytokines, etc.</th>
<th>Growth Factors</th>
<th>Kinases</th>
<th>Neurotransmitters</th>
<th>Receptors</th>
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</table>

Table 5. Various Mediators of Neuropathic Pain

**Brain & Brainstem**

Neuroimmune and neuroinflammatory mechanisms of neuropathic pain are least described in the brain. After rat CCI, microglial CD11b expression is increased in the hypothalamus and periaqueductal gray (Takeda et al., 2009). Memantine, an open-channel NMDAR antagonist, attenuated CD11b expression and allodynia (Takeda et al., 2009). The gracile nucleus increases microglial p-p38 after SNL injury that is reduced by analgesic p38 inhibitor into the cisterna magna (Terayama et al., 2008). In the case of a more proximal injury, trigeminal nerve injury, microglial and astrocytes reactivity is
observed in the medullary dorsal horn that is reduced by an analgesic minocycline therapy (Piao et al., 2006).

In summary, neuropathic pain is generally chronic with diverse etiology. Potential mechanisms of neuropathic pain are complex and numerous. The mechanisms broadly fall under the umbrella(s) of neuronal and inflammatory function. In recent years, the field has been (rightly) tightening up the use of terminology related to inflammatory processes, as microglial morphological changes do not constitute ‘inflammation’ or ‘neuroinflammation.’ Still, increasing evidence supports legitimate neuroinflammation in common models of neuropathic pain. The confirmation of some mechanisms in human neuropathic pain conditions are burgeoning as measures of neural activity improve (e.g., fMRI, central sensitization); the contribution of neuroinflammation will remain largely unknown until non-invasive techniques improve (e.g., imaging immune cell activity).

**Stress**

*Definition of Stress*

Like pain, stress is a subjective experience. A universal definition is lacking; however, a general consensus use of the term describes stress as the experience to a real or perceived threat to psychological or physiological homeostasis (McEwen, 2000). It follows, then, that a ‘stressor’ is distinguished as the threat, and the ‘stress response’ as the psychological and/or physiological reaction to the threat. It has been said that stressors
are adverse forces in the equilibrium, or homeostasis, of life (Tsigos and Chrousos, 2002). Hans Selye, who popularized stress research, defined stress as “the nonspecific response of the body to any demand” (Selye, 1976). Any experience can be interpreted as stressful. Not all stressors elicit a physiological stress response, nor is a cognitive appraisal required to trigger a physiological stress response. For the first case, experience (Cacioppo et al., 2000), genetics (DeRijk and de Kloet, 2005), coping skills and perception (Lazarus, 1993) influence the extent of stress response. To illustrate the latter, an episode of sufficiently disrupted physiological homeostasis (e.g., trauma) will elicit an endocrine stress response regardless of conscious awareness. This reinforces the evolutionarily conserved, protective purpose and physiological outcomes of stress system activation. In modern, industrialized society, however, most stressors are not life-threatening and produce unnecessary, even maladaptive stimulation of the endocrine stress response.

The physiological endocrine stress response is comprised primarily by constituents of the adrenal and autonomic systems. When the threat of a stressor emerges, the autonomic system rapidly responds. The locus ceruleus (LC) is the brain’s sympathetic axis effector; replete with norepinephrine (NE) stores, it responds rapidly to a threat by distributing NE via extensive cerebral projections (Berridge and Waterhouse, 2003). NE is also projected from the nucleus tractus solitarius of the medulla to the paraventricular nucleus of the hypothalamus (PVN) (Cunningham and Sawchenko, 1988; Palkovits et al., 1999). NE focuses attention (Alexander et al., 2007), enhances and/or impairs memory
(Arnsten and Li, 2005; Robbins, 2000; Sara, 2009), and decreases parasympathetic functions such as feeding and sleeping. Noradrenergic innervation of peripheral organs is initiated by cholinergic pre-ganglionic sympathetic neurons in the intermediolateral cell column of the thoraco-lumbar spinal cord. The adrenal medulla contributes considerable peripheral NE that is released on local post-ganglionic sympathetic neuron stimulation. Immune organs (e.g., spleen, thymus) receive noradrenergic innervations that are generally suppressive in nature for the innate system, and variable for the adaptive system (Nance and Sanders, 2007). Activation of the central and peripheral autonomic sympathetic axis is responsible for the ‘fight or flight’ aspects of the stress response. It was initially proposed by Walter B. Cannon that epinephrine was the primary sympathetic hormone of the adrenal gland (Cannon and Lissak, 1939), later to be corrected as NE (von Euler, 1946). Of pain and select emotions, Cannon described their role in sympathetic activation:

Pain, the major emotions—fear and rage—and also intense excitement, are manifested in the activities of the sympathetic division. When in these states impulses rush out over the neurones of this division they produce all the changes typical of sympathetic excitation, such as dilating the pupils, inhibiting digestion, causing pallor, accelerating the heart, and various other well-known effects (Cannon, 1915, p 35, 36).

Cannon was a pioneer in stress and physiology research; importantly, he coined the now widely-used term ‘homeostasis,’ as well as ‘fight or flight’ response (Cannon, 1915, 1932; Goldstein and Kopin, 2008).
Glucocorticoids are steroid hormones that are also primary effectors of the stress responses. In humans, the main GC is cortisol; whereas, in most rodents, it is corticosterone. Besides participating in conditions of disrupted homeostasis, GCs, like NE, have numerous regulatory physiological functions, including glucose metabolism. GCs derive from the adrenal cortex and exhibit a circadian, pulsatile rhythm, with widespread receptor (GR) localization. Stressors activate a circuit that results in GC release into the bloodstream. The circuit is comprised of the hypothalamus, pituitary gland, and adrenal cortex, and hence, termed the hypothalamic-pituitary-adrenal (HPA) axis. NE, cytokines and other agents stimulate corticotrophin-releasing hormone (CRH) release from the hypothalamic PVN. CRH is shuttled through the portal vasculature of the pituitary stalk to the anterior pituitary gland, where it stimulates corticotrope cells to release adrenocorticotropic (ACTH) hormone into the bloodstream. ACTH induces GC synthesis and release from the adrenal cortex. Entry into diverse cell types is facilitated by the lipophilic nature of steroid hormones. Some cell and receptor specific responses to GCs will be described in the following sections.

*Experimental Stress*

*Rodent Models*

There are numerous models of stress utilized in the basic laboratory setting. A sampling of experimental stressors is provided in Table 6. Important considerations for selecting a stressor include duration, frequency, and severity. These factors dramatically influence
outcome measures (Bowers et al., 2008; Dhabhar, 2000; Pacak and Palkovits, 2001; Pacak et al., 1998). Severity of stressor is conceptualized according to duration, and whether the stressor is primarily psychological, as in restraint, or physical and psychological (e.g., inescapable foot shock).

<table>
<thead>
<tr>
<th>Common Experimental Stressors</th>
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<tr>
<td>Rodent</td>
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<tr>
<td>Restraint</td>
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<td>Cold Water Swimming</td>
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<td>Social Defeat</td>
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<td>Foot/Tail Shock</td>
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<td>Heat Exposure</td>
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<td>Handling</td>
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<td>Noise</td>
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<td>Social Isolation</td>
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<td>Maternal Deprivation</td>
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<td>Predator Odor</td>
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<td>Sleep Deprivation</td>
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<td>Human</td>
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<td><strong>Laboratory</strong></td>
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<td>Trier Social Stress Test</td>
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<td>Public Speaking</td>
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<td>Mental Arithmetic</td>
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<td>Sleep Deprivation</td>
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<td><strong>Naturalistic</strong></td>
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<td>Academic Exams</td>
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<tr>
<td>Caregiving</td>
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<tr>
<td>Divorce</td>
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<td>Bereavement</td>
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</table>

Table 6. Experimental Stressors

Stress Effects on Nervous system

**Stress Effects on Functional Neuron Plasticity**

Stress and GCs may exacerbate neuropathic pain through modulation of neuroplasticity (Blackburn-Munro and Blackburn-Munro, 2003; Joels and Krugers, 2007; Revest et al., 2005; Wang et al., 2005b). In hippocampus and amygdala, stress and GCs facilitate long-term potentiation (LTP) or depression (LTD), depending on the type and duration of
stressor (Ahmed et al., 2006; Coussens et al., 1997; Diamond et al., 1992; Kim et al., 1996; Pavlides et al., 1993). Stress has been likened to LTP, whereby stress recruits shared signaling constituents and saturates LTP synaptic transmission, hence the common observation of LTD after stress (Huang et al., 2005). The use of LTP machinery by stress would transiently occlude LTP. Acute stress, for example, induces LTD and ERK1/2 phosphorylation that is reversible by MEK1/2, PKC or BDNF inhibition (Yang et al., 2004). Conversely, if stress promotes LTD directly and prior LTD facilitates subsequent LTP (Ngezahayo et al., 2000), then acute stress may prime synaptic potentiation. As such, acute stress also has been shown to induce LTP that was associated with a similar molecular signature of increased activated forms of ERK1/2, p38, CaMKII, and CREB (Ahmed et al., 2006). Stress-induced corticosterone may also contribute to mechanisms of LTP by increasing membrane trafficking of AMPA receptors (Groc et al., 2008).

Like NMDA receptor activity, ERK1/2 activation is a hallmark of synaptic plasticity and critical for LTP (English and Sweatt, 1996; Schafe et al., 2008; Toyoda et al., 2007) and behavioral measures of learning and memory (Atkins et al., 1998; Selcher et al., 1999). ERK1/2 activation is increased by stress after nerve injury (Alexander et al., 2009) and might be mediated by several factors. For example, stress has been shown to induce BDNF production (Yang et al., 2004; Yang et al., 2008). Corticosterone increases the excitability of basolateral amygdala cells by producing a depolarizing shift in the anion reversal potential (Duvarci and Pare, 2007), an observation akin to the pro-algesic effects of BDNF on lamina I nociceptive neurons (Coull et al., 2005). Stress induces BDNF
production downstream of GR activation (Yang et al., 2004; Yang et al., 2008), therefore corticosterone effects on chloride homeostasis may be related to BDNF. Stress also induces NMDA receptor activation (Ahmed et al., 2006; Kim et al., 1996; Norris and Strickland, 2007; Wang et al., 2005b; Yang et al., 2008). Corticosterone prolongs NMDA receptor-mediated Ca2+ elevation in hippocampal neurons in vitro (Takahashi et al., 2002) and acute stress enhances Ca2+ currents in vivo (Joels et al., 2003). In spinal cord dorsal horn neurons, activation of glutamate receptors is implicated in stimulation-evoked ERK1/2 phosphorylation (Daulhac et al., 2006; Ji et al., 1999; Karim et al., 2001; Kohno et al., 2008a; Lever et al., 2003). In support of the role of stress in neuroplasticity after injury, stress effects on neuropathic pain are prevented by NMDA receptor blockade (Alexander et al., 2009).

**Stress Effects on Structural Neuron Plasticity**

Structural plasticity, too, is apparent after stress. In the amygdala, acute stress activates ERK1/2 and GAP-43 to produce fear conditioning behavior associated with synaptic remodeling (Pawlak et al., 2003). Stress prior to axon injury increases subsequent neurite sprouting and elongation that is prevented by pre-stress treatment with memantine, an NMDA receptor antagonist (Chapter 3). Similarly, kainic acid and corticosterone treatment of dorsal root ganglia (DRG) neurons increases neurite outgrowth and GAP-43 expression (Tsai et al., 2002; Tsai et al., 2007). Stress hormones may, therefore, exert extrinsic modulation of injury-induced neuroplasticity at multiple levels to affect pain.
Stress Effects on Inflammatory Measures

Stress Effects on Immune Factors

Cellular constituents of the innate immune system are broadly responsive to stress hormones. Glucocorticoids are well-known for their immunosuppressive actions; however, GCs also can exert pro-inflammatory effects (Craft et al., 2006; de Pablos et al., 2006; Dinkel et al., 2003; Sapolsky et al., 2000). An important point of divergence in this paradoxical effect is often the source of GCs. Most commonly used synthetic GCs are more potent than the endogenous cortisol/corticosterone. This potency is nearly always associated with immunosuppression and anti-inflammatory effects (Dhabhar and McEwen, 1999), and largely attributed to actions on NF-κB signaling (Smoak and Cidlowski, 2004).

There are also reports of stress or endogenous GC induced immune suppression (Franchimont et al., 2002). Here, it seems that the major determinant of stress/GC effects on inflammation is duration or type of stressor (Bowers et al., 2008). Indeed, stress has paradigm-specific effects on inflammatory measures (Calcagni and Elenkov, 2006; Chrousos, 2000; Dhabhar, 2003). In general, acute stressors seem to increase the surveying capacity of the immune system. For example, acute restraint stress increases leukocyte trafficking to the skin (Dhabhar, 2000; Dhabhar and McEwen, 1999; Viswanathan et al., 2005; Viswanathan and Dhabhar, 2005) and may ‘ready’ microglia to respond to damage (Sugama et al., 2007). These preparations of the immune system by
stress may not constitute full-fledged inflammation but could explain exacerbated neurological conditions after stress. Unlike skin wounds that neatly proceed through stages of inflammation and resolution for healing, nervous system damage is not well-served by the same response (Popovich and Longbrake, 2008). For example, infarction volume and functional deficits in experimental stroke are increased by stress (Sugo et al., 2002). Likewise, stress exacerbates brain inflammation induced by LPS (de Pablos et al., 2006; Munhoz et al., 2006), and worsens pathology in rodent models of multiple sclerosis (Mi et al., 2006; Welsh et al., 2004). Acute psychological stress in humans increases circulating IL-6 and IL-1β (Steptoe et al., 2007) and exercise-induced cortisol increases human monocyte CCR2 expression and migration (Okutsu et al., 2008). These changes might be impactful to ongoing or ensuing inflammation.

**Stress Effects on Microglia**

Stress-induced activation of microglia is supported by *in vitro* studies that show functional glucocorticoid receptors (GR) and mineralocorticoid receptors (MR) on cultured microglia (Tanaka et al., 1997). Stress has been shown to exacerbate inflammation via stimulatory effects on inflammatory cells including microglia and mast cells. Restraint stress induces microglial cell proliferation and morphological changes in the central nervous system (Frank et al., 2006; Frank et al., 2010; Nair and Bonneau, 2006; Neigh et al., 2009; Sugama et al., 2007; Sugama et al., 2009). Restraint stress and exogenous corticosterone increase LPS-induced neurotoxicity and microgliosis in the brain that is prevented by treatment with RU486 (de Pablos et al., 2006; Frank et al., 2006).
Acute inescapable shock stress or corticosterone administration potentiates the microglial response to LPS 24 h later, *ex vivo* (Frank et al., 2006). In the absence of pathology, repeated stress induces microglia activation (Nair and Bonneau, 2006) and the expression of pro-inflammatory mediators in the CNS, including iNOS and TNF-α (Madrigal et al., 2002). Stress-induced release of pro-inflammatory cytokines, chemokines, nerve growth factor, free radicals, prostaglandins, and excitatory amino acids from immune cells are generally pro-algesic at sites of peripheral and central injury (Marchand et al., 2005; Watkins and Maier, 2002).

 Longer duration stressors (e.g., 12 h restraint, chronic unpredictable stress) are also often pro-inflammatory, increasing cytokines, chemokines, microglial phagocytosis capacity, and TLR expression (Munhoz et al., 2006; Shimoda et al., 2006). Chronic unpredictable stress potentiates pro-inflammatory effects of LPS (de Pablos et al., 2006). Repeated sound stress enhances hyperalgesia after an inflammatory stimulus (Khasar et al., 2008).

 In summary, the sympathetic and HPA axes response to a stressor promote adaptive physiological changes that support survival of the organism. Even with non-life threatening stressors, as are common in modern, industrialized society, acute and infrequent activation of these evolutionarily conserved responses are generally considered innocuous. Chronic exposure to stress hormones, however, has clear deleterious effects on health (Chrousos, 2009). It is intriguing to consider how the
physiological responses of the nervous and immune systems to acute stress might impact subsequent challenges, such as injury.

Proposed Role of Stress in Neuropathic Pain

We propose that stress-related hormones are a gateway by which variability (of severity or susceptibility) is introduced in clinical neuropathic pain. Endogenous GCs are pleiotropic, and thus uniquely capable of pro- and anti-inflammatory effects, neurostimulatory or inhibitory effects, as well as metabolic and cardiac effects. GCs cycle daily to support physiological demands, and rapidly increase in response to homeostatic disruption. GCs have easy access to otherwise guarded neural tissues. Hence, endogenous GCs are ideal candidates to set the tone and/or increase the gain on pain.

Our objective, as addressed in the following chapters, was to elucidate the role of endogenous GCs in experimental neuropathic pain. We hypothesized that endogenous GC spikes, as occur during stress or injury, facilitate the development of pain by exploiting cellular ‘survival’ mechanisms. In other words, while the stimulating effects of acute GC elevation on immune and neural systems are evolutionarily adaptive, they inadvertently enhance the potential for chronic pain. We predicted that the stress-primed immune system would generate a more robust response to nerve injury, and that either the magnitude of this response, or some factor in its initiation would be propagated to ultimately exacerbate pain. Acute stress has similar alerting effects on the neural system. We predicted that the molecular basis of stress-induced enhancement of attention and
memory would, in the context of nerve injury, translate into potentiated neural signaling that underlies pain processing. In essence, we suspect that stress converges on the same substrates affected by neural injury, and that this convergence can direct the susceptibility or severity of pain. Such a role for stress in sensory processing was previously unknown.

Figure 1. Potential Model of Stress Effects on Pain. Question #1 (Q1): Does stress and/or GCs increase neuropathic pain-like behavior? Question #2 (Q2): Does stress and/or GCs increase pain-like behavior via neuron or immune activity? For neurons, what type of activity is affected; for immune function, what cell type(s) is involved? Question #3 (Q3): What molecular substrates are used in these mechanisms?
Figure 1b. Alternative schematic for proposed stress effects on pain related to neuron plasticity.
Chapter 2: Stress Exacerbates Neuropathic Pain via Glucocorticoid and NMDA Receptor Activation

Introduction

Individuals suffering from cancer, stroke, spinal cord injury and multiple sclerosis often become debilitated by neuropathic pain. Pain likely develops as a consequence of enhanced neuro-immune signaling and central sensitization in the spinal cord (Campbell and Meyer, 2006; Ji and Strichartz, 2004; Tsuda et al., 2005). Since psychosocial stress is often endured alongside these conditions (Gold et al., 2005; Scadding and Koltzenburg, 2006; Strang, 1998) and clinical observations suggest that stress increases susceptibility to develop pain and exacerbates existing pain (Ashkinazi and Vershinina, 1999; DeLeo, 2006; Greco et al., 2004; Nicholson and Martelli, 2004; Turner et al., 2002), it is important to understand how stress affects the development and severity of neuropathic pain.

Stress hormones, e.g., glucocorticoids (GCs), could enhance pain-like behaviors (Blackburn-Munro and Blackburn-Munro, 2003). Conceptually, this may seem paradoxical because synthetic GCs are used to treat inflammatory conditions that cause pain including peripheral nerve injury. GCs, however, also play a central role in neuroplasticity (Joels and Krugers, 2007; Revest et al., 2005; Wang et al., 2005b). Glucocorticoid receptor (GR) expression is increased in spinal cord dorsal horn neurons
after nerve injury in rats (Wang et al., 2004; Wang et al., 2005b) and mice (Takasaki et al., 2005). Adrenalectomy (Wang et al., 2004) or intrathecal delivery of a GR antagonist (RU486) or GR anti-sense oligonucleotides reduces neuropathic pain caused by nerve injury (Takasaki et al., 2005; Wang et al., 2004). Also, intrathecal delivery of dexamethasone, a synthetic GC, exacerbates pain-like behaviors (Wang et al., 2004). Collectively, these data suggest that exogenous GCs can influence the onset or maintenance of neuropathic pain; however, the impact of stress has not been evaluated in this context.

The purpose of the current study was to test the novel hypothesis that stress increases neuropathic pain. The results show that stress potentiates nerve injury-induced tactile allodynia via a mechanism involving GCs acting at GRs and glutamate receptor-mediated ERK activation in dorsal horn neurons (Daulhac et al., 2006; Ji et al., 1999; Karim et al., 2001; Kohno et al., 2008a; Lever et al., 2003). Indeed, pERK was increased in the spinal cord dorsal horn in stressed/nerve-injured mice and pre-stress treatment with memantine, an NMDAR antagonist, prevented stress-enhanced allodynia. These novel data reveal that psychosocial stress, a natural consequence of most debilitating human diseases, can markedly exacerbate neuropathic pain and perhaps other pain-like syndromes (e.g., inflammatory pain) (Chover-Gonzalez et al., 2000; Khasar et al., 2008; Khasar et al., 2005; Rivat et al., 2007).
Methods

Animals

Adult female C57BL/6 mice (Taconic, Germantown, NY, USA) were group-housed in standard cages with *ad libitum* access to food and water. Mice were maintained in a vivarium with controlled temperature (~20º C) on a 12 hr light/dark cycle and were randomly assigned to experimental groups after a two week habituation period. Behavioral testing was performed during the light cycle. Mice were 8-10 weeks of age at the time of surgery. All procedures were conducted in accordance with protocols approved by the Ohio State University Institutional Laboratory Animal Care and Use Committee and with the guidelines of the Committee for Research and Ethical Issues of IASP.

Restraint stress protocol

Mice assigned to experimental groups incorporating stress were placed individually into well ventilated polypropylene tubes (2.8 cm internal diameter, 9.7 cm length) for 60 min. The restraint tube permits minimal, confined movement including postural adjustments. Mice not subjected to stress (No Stress groups) remained undisturbed in their home cages.

Drugs

Mifepristone (RU486; 50 mg/kg) and corticosterone (CORT; 1.5 mg/kg) were prepared in a sterile peanut oil vehicle (Veh). Memantine (MEM; 20mg/kg) was prepared in
sterile water vehicle. Drugs were administered in a 0.1 ml volume via intraperitoneal (i.p.) injection. All drugs were obtained from Sigma-Aldrich (St. Louis, MO, USA) and were prepared fresh daily. The concentration of CORT used in Experiment #2 was empirically defined to reproduce stress-induced plasma CORT concentrations in our lab and others (DeVries et al., 1995; Sugo et al., 2002). For in vitro experiments using CORT, two preparations were tested: CORT (0.1 μM) dissolved in 0.0005% DMSO and 0.005% EtOH and water-soluble CORT-2-hydroxymethyl-β-cyclodextrin (HBC) dissolved in media (both from Sigma). Lipopolysaccharide (LPS; E. coli 055:B5; lot no. 067K4056) was obtained from Sigma. Mouse recombinant tumor necrosis factor-α (rTNF-α) was obtained from eBioscience (San Diego, CA, USA).

Peripheral nerve injury procedure

The spared nerve injury (SNI) procedure was performed as described previously by Shields et al. (Shields et al., 2003). Under isoflurane anesthesia (4% induction, 1.5% maintenance) in oxygen-enriched air and following shaving and aseptic preparation, an incision of the skin and biceps femoris muscle was introduced to expose the sciatic nerve and its three terminal branches at the upper-thigh level. Two of the branches, the sural and common peroneal nerves, were tightly ligated with 7-0 silk suture (Genzyme Biosurgery, Fall River, MA) and transected distal to the ligature. Subsequently, 1-2 mm of each nerve was resected, approximately 1 mm distal to the ligature. The tibial nerve was not disturbed. The muscle/fascia layer and skin layer were closed separately with 5-0 nylon suture (Syneture, Norwalk, CT).
**Behavioral Testing**

Punctate mechanical sensitivity was analyzed by measuring threshold response to von Frey filaments (Stoelting Co., Wood Dale, IL). Monofilament stimulation was applied to the mid-line of the plantar surface of each hind paw using the up-down method for threshold sensitivity (Chaplan et al., 1994). The stimulus intensity threshold represents the smallest force that elicited repeated withdrawal of the hind paw during 10 trials (≥ 50% response sensitivity to smallest force). Threshold values represent von Frey hair handle markings that correspond to $\log_{10}$ of (10x force in mg), as displayed in Figs 2&7 (Chacur et al., 2001).

Behavioral assessments were made without stress prior to SNI (Baseline; BL), 1d after stress but prior to SNI (Pre-SNI) and on days 1, 3, 5, and 7d post- SNI (Post-SNI 1, 3, 5, 7; Table 1). Table 1 summarizes the pharmacological and behavioral paradigm utilized in these studies. The paradigm incorporates two stress sessions; however, a single session immediately prior to SNI is sufficient to enhance allodynia (data not shown).

Mice were acclimated to the testing procedure for 20 min each day for two days prior to the start of the experiment and for 10 min prior to the onset of each session. All behavioral measurements were conducted by an investigator that was unaware of the treatment groups.
Table 7. Description of Behavioral Experiments

<table>
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<tr>
<th>Experiment</th>
<th>Treatment</th>
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<tr>
<td>1 &amp; 4</td>
<td>No Stress or Stress (60 min, immediately prior to SNI)</td>
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<tr>
<td>2</td>
<td>Veh or RU486 (60 min prior to No Stress or Stress; Stress as above)</td>
</tr>
<tr>
<td>3</td>
<td>Veh or CORT (60 min prior to SNI)</td>
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<tr>
<td>5</td>
<td>Veh or MEM (60 min prior to No Stress or Stress; Stress as above)</td>
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**Determination of plasma corticosterone concentration**

At baseline (day -2 of the experiment), a blood sample was obtained from the periorbital sinus following restraint or injection of CORT. A sample also was collected from No Stress mice at baseline. On day 8 post-SNI, prior to transcardial perfusion, a blood sample was obtained from the periorbital sinus. All blood samples were collected within a 2 h time period beginning 3-5 h after the onset of the light cycle. After centrifugation, plasma was collected and stored at -70ºC. Levels of plasma CORT were measured in duplicate samples using an $^{125}$I radioimmunoassay kit (ICN Pharmaceuticals, Costa Mesa, CA).
**Histological analyses**

Mice were anesthetized with a ketamine/xylazine (80 mg kg$^{-1}$/10 mg kg$^{-1}$; i.p.) cocktail prior to transcardial perfusion with 30 ml 0.1 M phosphate-buffered saline (PBS, pH 7.4) followed by 100 ml of 4% paraformaldehyde (sample size for post-SNI tissue collection: n=4 for 1d; n=5-6 mice/group for 8d). After perfusion, spinal cords were rapidly removed then post-fixed for 2 h, followed by a rinse and overnight immersion in 0.2 phosphate buffer (PB) solution. Tissues were cryoprotected in 30% sucrose in 0.2 M PBS for 48 h. Spinal cords were blocked in 8 mm segments centered on the L-5 level, embedded in optimal cutting temperature compound (OCT; Tissue-Tek, VWR International, West Chester, PA) and frozen at -80ºC. Transverse serial sections (10 μm) were cut using a cryostat and thaw-mounted on SuperFrost Plus slides (Fisher Scientific, Houston, TX), then stored at -20ºC until use. After drying at room temperature, slides were rinsed with 0.1 M phosphate buffer solution (PBS) and overlaid with blocking solution for 1 h. To identify the central projections of injured axons, slides were processed for isolectin-B4 staining (IB4, *Griffonia simplicifolia*; Sigma; (Beggs and Salter, 2007)). After a brief 6% H$_2$O$_2$–MeOH incubation, slides were rinsed in 0.1 M PBS and incubated in Dulbecco’s PBS plus 0.1% Triton X-100 (Sigma) for 30 min, followed by biotinylated-IB4 (5 μg/ml) for 2 h at room temperature. After rinsing, slides were incubated in streptavidin-conjugated AlexaFluor 488. To label microglia, slides were incubated with anti-rabbit Iba-1 (Ito et al., 1998) (1:750; Wako, Richmond, VA) overnight at 4ºC. Slides were then rinsed in 0.1 M PBS and incubated with either biotinylated goat anti-rabbit antibody (Vector, Burlingame, CA) or AlexaFluor 546-
conjugated antibody in blocking solution. For immunoperoxidase labeling, slides were rinsed in 0.1 M PBS and incubated in 3% H$_2$O$_2$ to quench endogenous tissue peroxidase. Bound antibody was visualized using the Elite-ABC reagent (Vector) followed by DAB substrate (Vector). To label activated ERK, slides were processed for antigen retrieval according to the manufacturer’s instructions (Dako Target Retrieval Solution, Carpinteria, CA). Slides were incubated with anti-rabbit pERK (1:500; Cell Signaling Technology, Beverly, MA) overnight at 4°C. Sections were subsequently washed and incubated for 1-2 h with goat anti-rabbit AlexaFluor 546-conjugated antibody. While only 3-4mm of spinal cord is necessary for analyses of L4-L6 segments, an expanse of 8mm was collected initially to visualize any potential spreading of microglial reactivity beyond the affected segments. Loss of IB4 staining (Beggs & Salter, 2007; Shields et al., 2003) with a concurrent increase in morphological indices of microglial activation were used to identify spinal cord sections affected by injury. Analyses were conducted within this range of tissue sections. For analysis of labeling, the area containing the spinal cord dorsal horn was digitized under high-power using a Zeiss Axioplan 2 imaging microscope equipped with a digital camera (Zeiss Axiocam). Using an MCID Elite 6.0 image analysis system (Imaging Research, St. Catherines, Ontario, Canada), digitized images were quantified by outlining the superficial dorsal horn then quantifying areas of positive immunoreactivity (IR) relative to the total sample area, (expressed as proportional area (PA))(Kigerl et al., 2006). Iba-1 and p-ERK labeling were evaluated across the rostro-caudal extent of the 8mm segment (as described above); however, quantitative analyses were restricted to a series of five sequential sections (~200 μm
apart) centered on the area clearly affected by injury as defined above. All data were obtained by an investigator that was unaware of the treatment groups.

*Laser capture microdissection*

Mice were anesthetized as above and intracardially perfused at 3 days post-SNI with DEPC-treated 0.1 M PBS (n=5/group; No Stress and Stress). The lumbar enlargement of the spinal cord containing L₄-L₆ was blocked, embedded in OCT then flash frozen in N-methylbutane cooled by dry ice. Transverse serial sections (10 μm) were cut using a cryostat, then mounted on RNase free P.A.L.M. membrane slides (Zeiss, Thornwood, NY, USA) and stored at -80°C. The laser capture microdissection (LCM) procedure used here has been described previously by our lab (Kigerl et al., 2007; Longbrake et al., 2007). Briefly, sections were quick-stained with hematoxylin (Vector) and the dorsal horn region containing activated microglia (see Fig. 2) was circumscribed using the P.A.L.M. Microbeam laser capture system fit with a 5x cutting objective. Ipsilateral and contralateral dorsal horn tissue (~6 mm²) was collected separately in RNase-free tubes containing 20 μL of lysis buffer (RNaseous Micro kit, Ambion, Austin, TX, USA). RNA was isolated using the RNaseous Micro kit (Ambion) using the manufacturer’s protocol optimized for LCM samples. Samples were DNase treated (RNaseous kit, Ambion) and cDNA was prepared by reverse transcription with SuperScript II and random primers (Invitrogen, Carlsbad, CA, USA).
**Quantitative RT-PCR (qRT-PCR)**

To determine stress-induced effects on microglial activation and subsequent indices of neuroinflammation, select cytokine and microglial markers were analyzed from mRNA isolated from LCM samples. Gene-specific primer pairs for IL-1β, IL-6, TNF-α, and TLR4 were used as described previously (Kigerl et al., 2007). The primer pair for amplification of CD11b was forward 5’-GGATCATAGCGCCCCCACCT-3’ and reverse 5’-TCCTTACCCCCACTCAGGACT-3’; for IL-1α, forward 5’-CAGGATGTGGACAAACAC-3’ and reverse 5’-GCTCACGAACAGTTGTGAATCTG-3’. Gene expression was determined using qRT-PCR and compared between No Stress and Stress mice (n=4-5/group). Primer sequence specificity was confirmed by performing blast analysis for highly similar sequences against known sequence databases. PCR reactions were carried out in triplicate using 1μL cDNA/reaction and SYBR Green master mix (Applied Biosystems, Foster City, CA, USA) in 20 μL reactions. PCR product was measured using SYBR Green fluorescence collected on an Applied Biosystems 7300 system (Ririe et al., 1997). Standard curves were generated for each gene using a control cDNA dilution series. Melting point analyses were performed for each reaction to confirm single amplified products. ΔΔCt analysis was used to normalize gene data to 18s ribosomal RNA expression, then each ipsilateral value was expressed as fold change from its own contralateral value. This analysis was used for between-group comparisons (No Stress vs. Stress ipsilateral gene expression). Within-group comparisons reflect ipsilateral vs. contralateral (injury-induced) gene expression.
In vitro corticosterone experiments

The immortalized BV-2 microglia cell line was used to examine the priming effect of CORT on LPS or TNF-α–induced IL-1β gene expression. Cells were treated with CORT (0.1 μM or Veh) for 60 min or 24 h, then replaced with LPS (10 or 100 ng/ml, or Veh) or TNF-α (10 ng/ml, or Veh) for 60 min, 6 h or 24 h at 37ºC in a 5% CO2 humidified incubator. The Veh control for LPS and TNF-α was cell culture media (10% fetal bovine serum (Hyclone), 5% penicillin/streptamycin and 5% glutamax in DMEM (Gibco/Invitrogen). Independent studies have shown that GCs are not purely immunosuppressive; they can enhance certain immune functions, especially when present in low concentrations (Drew and Chavis, 2000; Lim et al., 2007; MacPherson et al., 2005; Zhu et al., 2007). Therefore, we compared varying CORT concentrations over a range from 0.1 μM to 1 mM (at 10X intervals) in an effort to identify an optimal priming dose. Since our goal was to prime rather than suppress microglial function, we used the lowest dose that did not suppress LPS-induced IL-1β gene expression. RNA was isolated using the Trizol method (Invitrogen) and IL-1β transcript was measured using qRT-PCR as described above. A minimum of three independent experiments was conducted for each in vitro manipulation.

To determine the capacity for CORT to prime intracellular signaling, thereby providing an explanation for stress-mediated increases in allodynia, we evaluated NF-κB signaling using 3T3 fibroblasts either transiently transfected or stably expressing an NF-κB responsive luciferase reporter (generously provided by Dr. Denis Guttridge). 3T3 stable

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cell lines were generated by seeding at a density of 2x10^5 cells in 6-cm dishes 24 h prior to transfection. Cotransfection of seeded fibroblasts with 4 ug of reporter plasmid 3κB-Luc and 1 ug of pcDNA3 (Invitrogen) was performed using Superfect reagent as recommended by the manufacturer (Qiagen). At 48 h post-transfection, the cells were trypsinized and cultured at 1/30 their density followed by selection in 1 mg/ml of Geneticin (G418; Life Technologies). Cells were allowed to expand under G418 selection and individual clones were selected as described by Guttridge et al. (Guttridge et al., 1999). Cells were treated with CORT (0.1 μM or Veh) for 60 min, then replaced with TNF-α (10 ng/ml, or Veh) for 60 min at 37°C in a 5% CO_2 humidified incubator. Transcriptional activity was measured in three independent experiments according to previously described methods (Guttridge et al., 1999).

Data analysis

Behavioral data for Experiments 1, 3, & 4 were analyzed using one-way repeated measures analysis of variance (ANOVA). Behavioral data for Experiments 2 & 5 were analyzed using two-way (drug x stress) repeated measures ANOVA. Post-hoc analyses were conducted using Bonferroni’s test. Factorial ANOVA was used for histological analyses. Student’s t test was used to analyze behavioral data for individual time points and between-group gene expression analyses. Within-group gene expression analyses were conducted using paired t-test. An α-level of p < 0.05 was used as an indication of statistical significance.
Results

Acute stress increases allodynia induced by nerve injury

To confirm that restraint stress elicited a physiologically appropriate response, we measured plasma CORT immediately after restraint. Circulating CORT was increased by ~400\% compared to the No Stress group ($F (1, 10)=16.97, p < 0.01$; $637.5 \pm 99.8$ ng/ml vs. $176.6 \pm 50.6$ ng/ml; $n=6$; Fig. 2A). Consistent with previous observations (Bomholt et al., 2005; Ulrich-Lai et al., 2006), CORT measurements 8d post-SNI revealed no stress- or injury-induced effects ($p > 0.05$; data not shown). In the absence of nerve injury, stress had no effect on paw withdrawal threshold ($p > 0.05$ vs. No Stress at Pre-SNI; Fig. 2B). Likewise, after sham surgery, stress had no effect on paw withdrawal threshold ($p > 0.05$ vs. No Stress; $n=2$; Fig. 2B). As expected, allodynia was observed following SNI as described previously ($F (1, 10)=103.51, p < 0.0001$ vs. Baseline (or vs. Sham: ($F (1, 14)=12.68, p < 0.01$) (Shields et al., 2003). This is a decrease in gram force threshold of ~80\% (0.3g vs. 1.5g) from Baseline. This pain-like response was consistently enhanced by stress ($F (1, 10)=87.20, p < 0.0001$ vs. No Stress; $n=6$; Fig. 2B).

RU486, a glucocorticoid receptor antagonist, mitigates stress-induced enhancement of allodynia after SNI

As in Experiment 1, allodynia was observed in all mice after SNI ($F (3, 16)=94.31, p < 0.0001$ vs. Baseline) and stress increased allodynia ($F (3, 16)=14.41, p < 0.0001$ vs. No Stress + Veh). To determine if stress-mediated increases in circulating CORT contribute
to enhanced allodynia, RU486 was injected prior to subjecting mice to stress and SNI. RU486 markedly suppressed allodynia in stressed mice ($p < 0.001$ vs. Veh; $n=5$/group; Fig. 2C) but had no effect on allodynia caused by nerve injury alone ($p > 0.05$ vs. Veh). At Baseline and Pre-SNI time points, there were no differences between groups ($p > 0.05$).

*Corticosterone increases nerve injury-induced allodynia*

We next tested whether exogenous CORT mimicked the effects of restraint stress. At Baseline or Pre-SNI time points, paw withdrawal thresholds were not different between CORT or Veh-treated mice ($p > 0.05$). However, 24 hours after administering exogenous CORT, paw withdrawal thresholds were reduced relative to Baseline values ($p < 0.0001$; baseline vs. pre-SNI, Figure 2D). Vehicle treatment had no significant effect on withdrawal threshold during pre-SNI testing ($p > 0.05$ vs. Baseline). After SNI, allodynia developed in all mice ($F (1, 8)=30.75$, $p < 0.0001$ vs. Baseline) but was exacerbated in mice treated with CORT prior to SNI ($F (1, 8)=16.58$, $p < 0.01$ vs. Veh; $n=5$/group; Fig. 2D).
Figure 2. Stress and corticosterone exacerbate nerve-injury induced allostynia. A) Acute stress (60 min restraint) increases plasma CORT (vs. No Stress (NS)). By binding GR, RU486 treatment increases plasma CORT (vs. Vehicle (Veh)). CORT treatment also increases plasma CORT (vs. Veh). B) Acute stress prior to SNI decreases the pain threshold (von Frey hair measurements) in the ipsilateral hind paw on post-SNI days 1, 3, 5, & 7 compared to the NS group. Stress has no effect on sham-operated mice (vs. NS; black circle = Sham, NS; white circle = Sham, Stress). C) When RU486 (RU) was used to block GRs, stress-induced potentiation of mechanical allodynia is reduced on post-SNI days 1 & 3 compared to Veh, as indicated by “##.” Asterisks (*) indicate the difference between Veh + NS and Veh + Stress allostynia on post-SNI days 1, 3, 5, & 7. D) Treatment with CORT prior to SNI decreases the pain threshold in the ipsilateral hind paw on post-SNI days 1, 3, 5, & 7 compared to Veh. Compared to Baseline (BL), CORT reduces pre-SNI thresholds (#p<0.0001). Results are expressed as the mean ± SEM; B.C.D; n=5-6/group SNI, n=2/group Sham; y-axis values represent von Frey hair handle markings. *p<0.05; ** or ##p<0.01; ###p<0.0001.

Activation of spinal cord dorsal horn microglia is accelerated by stress

In response to most types of peripheral nerve injury, spinal cord microglia change shape and increase expression of different cell surface antigens. These changes often predict the induction of pro-inflammatory functions in microglia that contribute to the onset and maintenance of pain-like behaviors (Narita et al., 2006c; Raghavendra et al., 2003; Tsuda et al., 2005). To determine whether this model of SNI consistently activates microglia and whether this response is exacerbated by stress, we first measured dorsal horn immunoreactivity for the microglial marker, Iba-1. In each experiment, SNI induced
morphological indices of microglial reactivity ipsilateral to the nerve injury ($t (8)=10.81$, $p < 0.0001$ vs. contralateral dorsal horn; Fig. 3A-D). Microglial activation was evident by 1 day post-lesion and increased further by 8 days (Fig. 3D). In stressed mice, microglial reactivity was increased at one day post-SNI ($t (6)=2.89$, $p < 0.05$, vs. No Stress; $n=4$/group; Fig. 3D); however, by 8 days, the effect of stress had dissipated ($n=5-6$/group; Fig. 3D).

Next, to better understand the potential functional implications of the enhanced morphological activation of microglia, we used laser capture microdissection to isolate injured and uninjured (contralateral) spinal cord dorsal horn regions populated by
activated and resting microglia, respectively, at 3 days post-SNI. From these samples we measured changes in expression of CD11b, TLR4, IL-1α, IL-1β, TNF-α, and IL-6 mRNA. Consistent with the data from Fig. 3, we found a small but non-significant induction of CD11b (a microglia-specific marker) mRNA after SNI (Fig. 4A) that was enhanced by stress ($t (4)=3.63, p < 0.05$ vs. contralateral; n=5/group; data not shown). Expression of CD11b and TLR4 in injured dorsal horn tissue was increased by stress ($t (7)=2.67, p < 0.05$ and $t (6)=4.03, p < 0.01$, respectively, vs. No Stress ipsilateral; n=4-5/group; Fig. 4A, B). However, expression of downstream cytokines, IL-1β, IL-6, TNF-α, and IL-1α was unaffected by SNI or stress ($p > 0.05$ for all comparisons; Fig. 4C-F).
Figure 4. Dorsal horn pro-inflammatory gene expression is not altered by stress 3 days post-SNI. A, B) Stress increases CD11b and TLR4 expression, indicative of enhanced microglial activation (also see Fig. 3). C-F) In contrast, the expression of pro-inflammatory genes known to be associated with neuropathic pain is not increased by SNI or Stress. Data are normalized to the internal control gene (18s), and expressed relative to their contralateral value. Results are expressed as mean ± SEM; n=4-5/group. *p<0.05; **p<0.01.

Corticosterone neither enhances nor suppresses inflammatory responses in vitro

Elevated transcript for CD11b and TLR4 (Fig. 4) suggests that microglia are primed by stress. The absence of stress-induced cytokines, however, may indicate that any priming effect of stress-derived glucocorticoids (GCs) on microglial function preceded the timing of our analyses (3 days post-SNI). Thus, two in vitro assays were used to test the
hypothesis that stress-induced GCs could prime intracellular signaling in microglia. Indeed, acute stress prior to surgery has been shown to enhance inflammation, and stress-induced CORT may alert immune cells to impending injury (Dhabhar, 2003; Viswanathan and Dhabhar, 2005).

First, to determine if GCs can enhance the function of microglia (e.g., in response to a stimulus released in dorsal horn after SNI), we examined the effect of CORT pre-treatment on BV-2 cells stimulated with LPS or TNF-α. LPS is the prototypical ligand for toll-like receptor 4 (TLR4), the activation of which contributes to cytokine production and pain after nerve injury (Tanga et al., 2005). TNF-α is produced after injury and also contributes to neuropathic pain (DeLeo et al., 1997; Hao et al., 2007; Schafers et al., 2003a; Schafers et al., 2003b; Zanella et al., 2008). Further rationale for the use of LPS and TNF-α derives from the observed stress-induced increase in TLR4 (LCM data; Fig. 4), that, when activated, drives NF-κB activation and subsequent TNF-α production. When CORT was applied to cells for 1 or 24 hours (based on the in vivo stress and CORT pre-treatment paradigm and time course of development of allodynia, respectively; see Fig. 2) then replaced with LPS or TNF-α for 1, 6 or 24 hours, IL-1β gene expression was generally unaffected (p > 0.05 vs. Veh pre-treatment). In one out of three experiments, CORT pre-treatment increased IL-1β mRNA; however, the reason for the inconsistency could not be determined and no contamination was evident. Because ethanol, a component of the vehicle in which CORT was prepared, suppresses TLR4 function (Dai and Pruett, 2006), we next tested water-soluble CORT. Again, CORT pre-treatment had
no effect on LPS- or TNF-α-induced IL-1β gene expression ($p > 0.05$ vs. Veh+LPS/TNF-α; Fig. 5A). LPS-induced nitric oxide production was also unaffected by CORT pre-treatment (data not shown).

To broaden the analysis further, we analyzed CORT effects on priming of NF-κB transcription. NF-κB operates upstream of numerous pro-inflammatory mediators that are known to cause or be associated with pain (Meunier et al., 2007; Tegeder et al., 2004). NIH3T3 cells were either stably or transiently transfected with an NF-κB responsive luciferase reporter, 3XκB. Cells were primed with CORT for 1 hour then were stimulated with TNF-α (10ng/ml) for 1 hour. Transcriptional activity was measured by luciferase assay as previously described (Guttridge et al., 1999). Although TNF-α markedly enhanced NF-κB activity ($p < 0.05$ vs. Med/CORT Veh), CORT pre-treatment did not increase (or decrease) this response ($p > 0.05$ vs. Veh + TNF-α; Fig. 5B).
Figure 5. Glucocorticoids do not prime pro-inflammatory gene expression or NF-κB activity. A) LPS (10 and 100 ng/ml) and TNF-α (10 ng/ml) for 1 or 6h increase IL-1β transcript (vs. media) in BV-2 cells. Pre-treatment with CORT (0.1 μM) for 1h has no effect on LPS- or TNF-α-induced IL-1β expression. B) TNF-α (10 ng/ml) for 1h increases NF-κB activity (vs. media & CORT vehicle). Pre-treatment with CORT (0.1
μM) for 1h has no effect on TNF-induced NF-kB activity. Relative luciferase activity (RLA) was determined by normalizing to b-gal. Results are expressed as mean ± SEM. *p<0.05; **p<0.0001.

**Stress increases phosphorylation of ERK in the dorsal horn after SNI**

Because stress-enhanced allodynia was not accompanied by a persistent pro-inflammatory microglial response, we next questioned whether the effects of stress were a result of enhanced central sensitization. Indeed, acute stress and GCs can sensitize neurons to injury-induced activation in part by increasing glutamatergic signaling via NMDA receptors (Ahmed et al., 2006; Kim et al., 1996; Norris and Strickland, 2007; Wang et al., 2005b; Yang et al., 2008). Activation of glutamate receptors has been implicated in stimulation-evoked ERK activation in spinal cord dorsal horn neurons (Daulhac et al., 2006; Ji et al., 1999; Karim et al., 2001; Kohno et al., 2008a; Lever et al., 2003), therefore we determined the effect of stress on dorsal horn pERK immunoreactivity. As shown in Fig. 6, stress increased pERK labeling in the superficial laminae one day after SNI (t (38)=4.58, p < 0.05 vs. No Stress; n=4/group). Consistent with previous work (Ji et al., 1999; Song et al., 2005; Zhuang et al., 2005), pERK-positive cells exhibited neuronal morphology.
Figure 6. Stress increases nerve injury-induced p-ERK. A&B) pERK labeling in superficial dorsal horn at 1 day post-SNI is increased by stress. C) Quantitative analysis of pERK labeling confirms stress-induced increases in pERK (p < 0.0001 vs. No Stress (NS); PA=proportional area). Results are expressed as mean ± SEM; n=4/group. Scale bar in B = 100μm.

Memantine blocks stress-enhanced alldynia after SNI

To determine whether the stress-mediated increase in pERK predicts or reflects enhanced signaling via NMDA receptors and thus “central sensitization”, we pre-treated mice with memantine (MEM), a moderate affinity NMDA receptor antagonist. MEM pre-treatment attenuated the development of allodynia in both Stress and No Stress mice; however, this effect was only evident at 1 day post-SNI (p < 0.01 vs. Veh). In contrast, MEM prevented the enhanced pain-like behavior caused by stress for the duration of the testing (F (3, 15)=9.61, p < 0.001 vs. Veh+Stress; n=6-7/group; Fig. 7).
Figure 7. Stress-induced potentiation of allodynia is NMDA receptor-dependent. Pre-stress treatment with memantine (MEM) blocks the effects of stress on allodynia (post-SNI days 1, 3, 5, and 7) after nerve injury (\*\*p<0.01 vs. Veh + Stress). Results are expressed as mean ± SEM; n=6-7/group; y-axis values represent von Frey hair handle markings.

Discussion

The present data show that stress exacerbates pain-like behavior caused by peripheral nerve injury. Two lines of evidence reveal a causal role for glucocorticoids (GCs) in this response. First, exogenous CORT reproduced the effects of stress. Second, pharmacological inhibition of glucocorticoid receptors (GRs) with RU486 blunted the potentiating effects of stress on nerve injury-induced allodynia. Recently, it was shown that nerve injury increases spinal GRs (Takasaki et al., 2005; 2006; Wang et al., 2004; Wang et al., 2005b). Thus, the potential for stress-induced GCs to exert effects on spinal cord neurons or glia is enhanced by nerve injury. Despite the novelty of these data, the precise cellular and molecular intermediates affected by circulating GCs that results in
exacerbated neuropathic pain are unknown. We tested two distinct mechanisms of neuropathic pain as substrates for CORT effects: microglial reactivity and neuronal sensitivity.

Acute stress activates inflammatory processes (Burns et al., 2008; Steptoe et al., 2007; Viswanathan and Dhabhar, 2005) and neuroinflammation has been implicated in the induction of neuropathic pain in models of central and peripheral nervous system injury. Therefore we predicted that resident CNS “immune cells”, i.e., microglia (Gehrmann et al., 1995; Streit et al., 1988), would exist in a hyperactivated state in stressed mice. Although we observed that stress accelerated morphological (Iba-1 labeling) and phenotypic (CD11b and TLR4 mRNA) indices of microglial activation elicited by SNI, and that these changes temporally corresponded with the onset of an exaggerated allodynia (~24-72 hours post-SNI), these changes were not maintained. Moreover, we found no evidence that stress or GCs could enhance pro-inflammatory signaling in vivo or in vitro. It is possible that had we used primary microglia instead of BV-2 cells, GC-mediated priming would have become evident. Indeed, Horvath et al. (2008) have shown that compared to BV-2 cells, primary microglia are more responsive to pro-inflammatory signaling. Still, we found little evidence of enhanced microglial activation or cytokine signaling in vivo (see above and Fig. 4). Collectively, these data were surprising given that there is a large body of evidence linking microglia and pro-inflammatory cytokines (mRNA and protein) to the induction of neuropathic pain (DeLeo and Yezierski, 2001; Detloff et al., 2008; Milligan and Watkins, 2009; Narita et al., 2006c; Tsuda et al., 2005).
However, independent studies have shown that acute stress, akin to that used in this report, can induce rapid (within 6 hours) and transient morphological changes in microglia without evoking changes in pro-inflammatory cytokines (Sugama et al., 2007).

Although stress accelerated the microglial response to SNI without an apparent increase in expression of pro-inflammatory cytokine mRNA or NFκB transcription, we still cannot entirely dismiss a role for activated microglia in contributing to the enhanced pain-like state experienced by stressed mice. Indeed, changes in mRNA do not always faithfully predict changes in synthesis or release of that protein. Also, microglia release other mediators, including brain derived neurotrophic factor (BDNF), that we did not measure but that can enhance neuronal excitability. In response to nerve injury, activation of purinergic receptors (e.g., P2X4) on microglia elicits BDNF release, which in turn increases neuronal excitability (Coull et al., 2005; Tsuda et al., 2003; Ulmann et al., 2008). Interestingly, enhancement of BDNF synthesis is observed following acute stress (Smith et al., 1995; Yang et al., 2008). To our knowledge, the regulation of BDNF production after stress or in response to exogenous GCs has not been established in the spinal cord after nerve injury. Thus, it is feasible that GCs facilitate microglia-neuron communication via BDNF. This could facilitate or act in parallel with stress and GC-mediated activation of mitogen activated protein kinases (MAPKs) (Meller et al., 2003; Norris and Strickland, 2007; Revest et al., 2005; Sananbenesi et al., 2003) and NMDA receptor activation in neurons (Cho and Little, 1999; Jing et al., 2008; Qi et al., 2005; Wang et al., 2005b; Wong et al., 2007; Yang et al., 2008). Other glial cells, particularly
astrocytes, have well-documented roles in neuropathic pain (Kawasaki et al., 2008; Tanga et al., 2004; Zhuang et al., 2006) and are responsive to corticosterone exposure (Melcangi et al., 1997; Ovadia et al., 1984; Vielkind et al., 1990), thus could play a role in stress-induced pain enhancement (LaCroix-Fralish and DeLeo, 2007).

Most of the data in this report suggest that the combination of stress and nerve injury triggers a complex interplay between GCs, GRs and enhanced glutamate transmission. Specifically, the stress-induced increase in activation of extracellular signal-regulated protein kinase (pERK) in spinal cord dorsal horn and the ability of memantine, an open-channel NMDA receptor antagonist, to block stress-enhanced allodynia (see Figs. 6&7), suggest that these pathways converge to cause central sensitization. Published data support this notion. Neuronal and glial glutamate transporters in the spinal cord are downregulated and glutamate uptake is reduced after nerve injury (Binns et al., 2005; Hughes et al., 2004; Sung et al., 2003). Importantly, EAAC1 downregulation is regulated by GRs; RU486 or GR antisense oligonucleotide treatment restores EAAC1 expression after nerve injury (Wang et al., 2006). These data support the possibility that stress-induced activation of GR results in decreased glutamate uptake and subsequent NMDA receptor activation. Indeed, stress or corticosterone has been shown to decrease glutamate transporter expression (Jacobsson et al., 2006; Madrigal et al., 2003) and increase extracellular glutamate (César Venero, 1999; Lowy et al., 1993; Moghaddam et al., 1994; Reznikov et al., 2007). GCs also augment nerve injury-induced expression of neuronal NMDA receptors, a phenomenon reversible by RU486 (Wang et al., 2004). Activation of
NMDA and metabotropic glutamate receptors are implicated in ERK activation in dorsal horn spinal cord neurons and the subsequent induction of pain (Daulhac et al., 2006; Ji et al., 1999; Karim et al., 2001; Kawasaki et al., 2004; Kohno et al., 2008a; Lever et al., 2003; Xu et al., 2008). Thus, suppression of neuronal and glial glutamate transporters by stress and GCs could impair removal of glutamate from the extracellular space and prime glutamatergic transmission through MAPK activation and upregulation of ionotropic and/or metabotropic glutamate receptors.

Clearly, more work is needed before we fully understand the mechanisms underlying the exaggerated pain-like behavior that we observed in stressed mice. However, this study has important clinical implications as the data show that stress may predict the induction and pathogenesis of chronic pain (Diatchenko et al., 2006). The significance of psychosocial effects on pain is emphasized by the delayed onset of neuropathic pain in various diseases and neurological disorders including cancer, post-herpetic neuralgia, amputation, HIV, ischemia and spinal cord injury. This temporal gap between insult and pain onset provides an opportunity for clinical intervention and underscores the need for greater understanding of the mechanisms that control communication between stress hormones, neurons and CNS glia.
Chapter 3: Nerve regeneration and sensory axon sprouting is increased by stress and is dependent on glucocorticoid receptor and mTOR

Introduction

Nervous system injury activates intrinsic mechanisms of neuroplasticity. The neuronal reaction to peripheral nerve damage is robust, and may promote axon regeneration, arborization, reorganization, or synaptic remodeling. Possible outcomes of these structural changes include motor and/or sensory recovery, pain and spasticity. At present, the conditions that direct these disparate outcomes are unknown. Indeed, there is marked variability in the human response to peripheral nerve damage and there are no known markers that predict recovery or neuropathic pain. Acute elevation of glucocorticoids (GCs), as occurs during stress, exercise, surgery and injury is a potential modulator of neuron plasticity (Alexander et al., 2009).

The role of stress and GCs in spinal and peripheral nervous systems is largely unknown; however, in multiple brain regions, stress and GCs robustly alter neuroplasticity. This is best described for measures of structural, biochemical, and functional (e.g., LTP, LTD) plasticity in cortical and subcortical structures responsible for behavior and cognition (Ahmed et al., 2006; Coussens et al., 1997; Diamond et al., 1992; Duvarci and Pare, 2007; Groc et al., 2008; Joels and Krugers, 2007; Joels et al., 2003; Kim et al., 1996; Pavlides et al., 1993; Revest et al., 2005; Takahashi et al., 2002; Yang et al., 2004). Fear
conditioning behavior, for example, is elicited by acute stress that is associated with activation of ERK1/2, GAP-43 and synapse remodeling in the amygdala (Pawlak et al., 2003). Likewise, acute stress or corticosterone exposure increases dendritic arborization of amygdala (Mitra and Sapolsky, 2008) and hippocampal neurons (Kole et al., 2004).

Functional outcomes of dendrite expansion or synapse formation in the brain include effects on learning, memory, fear, anxiety, and other behaviors. Sensory axon sprouting is associated with the development of pain after injury (Di Sebastiano et al., 1997; Fried and Devor, 1988; Gilmer-Hill et al., 2002; Woolf et al., 1992; Woolf et al., 1995). After spinal cord injury, axon sprouts exhibit calcitonin gene-related peptide (CGRP) immunoreactivity and terminate in the superficial dorsal horn, suggesting formation of functional pain fibers (Ondarza et al., 2003). Likewise, sprouting of small diameter C fibers into deeper dorsal horn laminae indicates nociceptor reorganization into low threshold pathways, which may underlie allodynia (Hu et al., 2004; Woolf et al., 1992; Woolf et al., 1995).

Stress effects on sensory axon plasticity are unknown; however, the concept is supported by the observation that acute stress prior to peripheral nerve injury increases dorsal horn p-ERK and neuropathic pain (Alexander et al., 2009). Stress and GCs may increase neuropathic pain by altering sensory neuron plasticity. Indeed, a role for steroid hormones in axon growth has recently been identified, wherein estrogen (Chakrabarty et
al., 2008) and GCs in combination with kainic acid (Tsai et al., 2002; Tsai et al., 2007) increase sensory axon outgrowth. Kainic acid and corticosterone treatment of cultured dorsal root ganglia (DRG) neurons increases GAP-43 expression and neurite outgrowth (Tsai et al., 2002; Tsai et al., 2007). Stress hormones may, therefore, impact injury-induced neuroplasticity at multiple levels to affect pain and axon regeneration (Blackburn-Munro and Blackburn-Munro, 2003; Revest et al., 2005; Takasaki et al., 2005; Wang et al., 2004; Wang et al., 2005b). Because stress is a physiological stimulus for GCs that is mimicked by trauma, exertion, and disease, we propose that extrinsic modulation of sensory circuitry by GCs is of global importance in conditions that precipitate nerve regeneration and pain.

Methods

Animals

Adult female C57Bl/6 mice (8-10 weeks; Taconic, Germantown, NY, USA) were group-housed in standard cages with ad libitum access to food and water. Mice were maintained in a vivarium with controlled temperature (~20º C) on a 12 hr light/dark cycle and were randomly assigned to experimental groups after a one week habituation period. All procedures were conducted in accordance with protocols approved by the Ohio State University Institutional Laboratory Animal Care and Use Committee and with the guidelines of the Committee for Research and Ethical Issues of IASP.
Restraint stress protocol

Mice assigned to experimental groups incorporating stress were placed individually into well-ventilated polypropylene tubes (2.8 cm internal diameter, 9.7 cm length) for 60 min. The restraint tube permits minimal, confined movement including postural adjustments. Mice not subjected to stress (No Stress groups) remained undisturbed in their home cages.

Drugs

Mifepristone (RU486; 50 mg/kg) and corticosterone (CORT; 1.5 mg/kg) were prepared in a sterile peanut oil vehicle (Veh) and administered in a 0.1 ml volume. The concentrations of injected CORT were previously determined to reproduce stress-induced plasma CORT concentrations (Alexander et al., 2009). Memantine (MEM; 20mg/kg) was prepared in sterile water vehicle. RU486, CORT and MEM were obtained from Sigma-Aldrich (St. Louis, MO, USA). Rapamycin (4 mg/kg; Tocris, Ellisville, MO) was prepared in 3% DMSO in sterile PBS, and administered in a 0.3 ml volume. All drugs were prepared fresh daily and delivered via intraperitoneal (i.p.) injection.

In vitro DRG experiments

Mouse DRGs were harvested after 60 min restraint (Stress) or without restraint (No Stress), as described previously (Gensel et al., 2009). Briefly, single cell suspensions of cervical, thoracic and lumbar DRG neurons were prepared from anesthetized adult (2-3 mos.) mice. Dissected DRGs were incubated in dispase 2 (5 U/ml; Roche, Penzbeg,
Germany) and collagenase type 2 (200U/ml; Worthington, Lakewood, NJ) for 45-60 min at 37°C in Hanks' Balanced Salt Solution (HBSS; Mediatech, Herndon, VA), followed by DNase 1 type 2 (250 µg/ml; Sigma) treatment for 5 min. Next, DRGs were triturated in 500 ml HBSS media through fire-polished Pasteur pipettes and spun at 3000 rpm for 3 min. The neuron-enriched pellet was re-suspended in 100 ml of Neurobasal A media supplemented with 2% B27, 1% Glutamax™, and 1% penicillin-streptomycin. All media components were obtained from Invitrogen (Gland Island, NY). Neurons were plated onto coverslips at 400 cells/coverslip, or 10K/coverslip for high-density growth analysis. All coverslips were pre-coated with 0.1 mg/ml of poly-L-lysine (Sigma) and 10 µg/ml of laminin (Invitrogen). After designated growth periods at 37°C in a 5% CO2 humidified incubator, cells were fixed with 2% paraformaldehyde for subsequent immunohistochemistry, or collected in M-PER for protein isolation and subsequent Western blot procedures.

**Measures of in vitro axon growth**

A monoclonal anti-b-tubulin III antibody was used to label DRG neurons (1:2000; Sigma). Alexa Fluor 546-conjugated secondary antibodies (1:1000) were used to detect labeled DRG neurons. Four to six coverslips were generated for each experimental condition. A minimum of three independent experiments were conducted for each *in vitro* manipulation. To quantify axon density per coverslip, automated sampling was performed to randomly sample 45% of the coverslip (~100 fields; MCID 6.0 Elite). Only samples that contained at least one soma bearing at least one neurite greater than the
diameter of the soma were digitized. Positive immunoreactivity for β-tubulin III was quantified per soma for each field using densitometric thresholds, excluding the area representing soma. To quantify the growth of individual neurons, isolated neurons were digitized using 10x or 20x objectives. To reduce human bias, an automated Sholl (1953) analysis program was developed using the MetaMorph image analysis system, as described previously (Gensel et al., 2009). Briefly, templates of concentric circles of 50mm intervals were overlaid onto the center of a digitized DRG soma. For each neuron, densitometric thresholds were set to remove background labeling and identify detailed cellular processes. The total number of objects above threshold intersecting each circle was tallied using an automated macro. The maximal ring with an intersecting process (max distance) and sum of the number of intersections (branching complexity) for all rings were generated for each cell and compared among groups.

**Immunohistochemistry**

Mice were anesthetized with a ketamine/xylazine (80 mg kg$^{-1}$/10 mg kg$^{-1}$; i.p.) cocktail prior to transcardial perfusion with 30 ml 0.1 M phosphate-buffered saline (PBS, pH 7.4) followed by 100 ml of 4% paraformaldehyde (sample size for post-SNI tissue collection: n=4 for 1d; n=5-6 mice/group for 8d). After perfusion, sciatic nerves were rapidly removed then post-fixed for 2 h, followed by a rinse and overnight immersion in 0.2 phosphate buffer (PB) solution. Tissues were cryoprotected in 30% sucrose in 0.2 M PBS for 48 h. Nerves were embedded in optimal cutting temperature compound (OCT; Tissue-Tek, VWR International, West Chester, PA) and frozen at -80°C. Longitudinal
serial sections (10 μm) were cut using a cryostat and thaw-mounted on SuperFrost Plus slides (Fisher Scientific, Houston, TX), then stored at -20°C until use. After drying at room temperature, slides were rinsed with 0.1 M phosphate buffer solution (PBS) and overlaid with blocking solution for 1 h. To label SPRR1A, slides were incubated with anti-rabbit SPRR1A (1:1000; gift from Dr. Strittmatter) overnight at 4°C. Sections were subsequently washed and incubated for 1-2 h with goat anti-rabbit AlexaFluor 546-conjugated antibody. DAPI was applied in the final rinse to visualize nuclei. Increased cellularity in the nerve, as indicated by DAPI, was used to identify the proximal aspect of the nerve lesion. Analyses were conducted from this proximal point to the most distal aspect of the nerve section. Five sections equally distributed across the extent of the nerve were analyzed per animal. For analysis of labeling, the area described was digitized under high-power using a Zeiss Axioplan 2 imaging microscope equipped with a digital camera (Zeiss Axiocam). Using an MCID Elite 6.0 image analysis system (Imaging Research, St. Catherines, Ontario, Canada), digitized images were quantified by outlining the nerve area described, then quantifying areas of positive SPRR1A immunoreactivity (IR) relative to the total sample area, (expressed as proportional area (PA))(Kigerl et al., 2006). All data were obtained by an investigator that was unaware of the treatment groups.

**Western Blot**

Cell lysates were prepared from DRG neurons by homogenizing in 250 μl T-PER with protease and phosphatase inhibitors additives (ThermoScientific, Rockford, IL)
immediately after harvest. Each sample was centrifuged (10 000 x g for 10 min) and the supernatant aliquoted to produce two separate blots, one designated for incubation with antibodies against the total signaling protein and one with antibodies against phosphorylated protein. Total protein concentration was determined using a BCA protein assay kit (ThermoScientific). Unless specified, antibodies were procured from Cell Signaling Technology (Beverly, MA). Samples (10 μg per well) were separated on 10% Bis-Tris gels and transferred to a PVDF membrane in a wet-transfer apparatus (Invitrogen). After protein transfer, membranes were incubated with 5% BSA for 1 h at room temperature, then with primary antibodies (1:1000-1:2000) in 5% BSA at 4°C for at least 12 h, and finally with secondary antibodies (1:5000-1:15,000) in 5% BSA for 1 h at room temperature. The membrane was washed three times with PBS + 5% Tween for 10 min each between incubations. HRP activity was visualized using a chemiluminescent substrate and signal density quantified with a Kodak Image Station 4000MM Pro (Carestream Health, Inc., Rochester, NY).

Sciatic Crush Injury
The sciatic crush injury procedure was performed under isoflurane anesthesia (4% induction, 1.5% maintenance) in oxygen-enriched air and following shaving and aseptic preparation. A right unilateral sciatic crush was performed at upper-thigh level for 20 s with a sterile curved micro-aneurysm clip. The clip is factory calibrated to produce a clamping force of 85 gm (Harvard Apparatus, Holliston, MA). This duration of crush
does not produce foot drop post-operatively. The muscle/fascia layer and skin layer were closed separately with 5-0 nylon suture (Syneture, Norwalk, CT).

**Behavioral Testing**

Punctate mechanical sensitivity was analyzed by measuring threshold response to von Frey filaments (Stoelting Co., Wood Dale, IL). Monofilament stimulation was applied to the mid-line of the plantar surface of each hind paw using the up-down method for threshold sensitivity (Chaplan et al., 1994). The stimulus intensity threshold represents the smallest force that elicited repeated withdrawal of the hind paw during 10 trials (≥ 50% response sensitivity to smallest force). Threshold values represent von Frey hair handle markings that correspond to \( \log_{10} \) of (10x force in mg), as displayed in (Chacur et al., 2001).

Thermal sensitivity was tested using the Hargreaves’ method (Hargreaves et al., 1988) using the Plantar Test Apparatus (Ugo Basile, Comerio, Italy), as described previously (Hoschouer et al., 2010). The latency to withdraw the hind paw from the infrared radiant heat stimulus (25 W) is recorded. Both hind paws were tested for five trials in a randomized order with at least one minute between trials. To avoid tissue damage, the heat stimulus is disengaged after 30 sec for any non-responsive mice. The average duration across trials was used for data analyses.
Mice were acclimated to the testing procedure for 20 min each day for two days prior to the start of the experiment and for 10 min (von Frey) or 40 min (Hargreaves’) prior to the onset of each session. von Frey and Hargreaves’ measurements were conducted on different days to avoid overstimulation of the hind paws. All behavioral measurements were conducted by an investigator that was unaware of the treatment groups.

Statistics
Behavioral data were analyzed using repeated measures analysis of variance (ANOVA). Factorial ANOVA was used for histological analyses for comparisons of more than two groups. Post-hoc analyses were conducted using Bonferroni’s test. Student’s t test was used to analyze histology comprising only two groups. An α-level of $p < 0.05$ was used as an indication of statistical significance.

Results
Sensory neurons express abundant glucocorticoid receptor
Stressors elicit numerous physiological changes; among the most prominent is activation of the hypothalamic-pituitary-adrenal (HPA) axis that results in increased circulating GCs. We hypothesize that stress-mediated increases in GC release could induce plasticity via GR-dependent mechanisms. Few studies have examined the effects of GCs on sensory processing pathways and the relative abundance of GR is unknown compared to GR-enriched tissue regions (e.g., hippocampus). To determine the relative content of neuronal GR, we performed Western blot analyses and
immunohistochemistry for cellular localization. Compared to hippocampus, mouse DRG demonstrates elevated GR (p < 0.001 for each; Fig. 8A) that localizes with neurons in vitro and in vivo (Fig. 8B & C). Therefore, stress likely plays a previously unappreciated role in these areas replete with GR.

Figure 8. Dorsal root ganglia neurons express abundant levels of glucocorticoid receptor. To determine the expression of DRG GR relative to areas known to express abundant GR, we compared tissue homogenates from hippocampus and DRG. A) Western blot analyses for GR. DRG expression of GR exceeds hippocampus (p < 0.0001). B) Localization of GR to neurons in DRG tissue. C) Localization of GR to DRG neurons in vitro. Scale bar = 40 μm.
Stress increases sensory axon sprouting and elongation

Treating DRG neurons in vitro with kainic acid and corticosterone together increases axon growth (Tsai et al., 2002; Tsai et al., 2007). A physiological stressor is associated with both increased corticosterone and extracellular glutamate. To determine if stress in vivo increases axon growth, mice were subjected to restraint stress for 60 min immediately prior to DRG harvest. The effects of stress were measured after 72 h in culture. Stress prior to DRG harvest increased axon growth (p < 0.05 vs. No Stress; Fig. 9A-C). High-density neuron cultures support enhanced axon growth compared to low-density cultures; however, because of axon overlap, neurons plated at higher density obscure details of axon length and sprouting per cell, classifications that bear functional importance (Bomze et al., 2001). To identify the growth phenotype promoted by stress, single DRG neurons were also examined. Stress increased both measures of axon sprouting (p < 0.0001 vs. No Stress) and long-distance axon growth (p < 0.05 vs. No Stress; Fig. 9D-F).
Figure 9. Stress prior to sensory neuron injury increases structural plasticity. To determine if stress influences axon growth after axotomy, mice were subjected to restraint stress for 60 min immediately prior to DRG harvest. A-C) High-density neuron cultures analyzed after 72h in vitro show enhanced axon growth density by stress (p < 0.05 vs. No Stress). D-F) To identify the growth phenotype promoted by stress, single DRG neurons were examined after 72h growth. F) Stress increased composite growth parameters of axon length and sprouting (p < 0.0001 vs. No Stress). F inset) Stress increases sprouting-like growth, demonstrated by branching complexity (p < 0.0001 vs. No Stress). Neuron images represent statistical mean. Results are expressed as mean ± SEM. Scale bar (E) = 20 μm. *p<0.05; ***p<0.0001.

**Stress-induced sensory neuron plasticity is mediated by corticosterone**

To determine if stress-induced enhancement of axon growth after nerve injury is mediated by GCs, mice were treated with CORT (1.5 mg/kg) prior to DRG harvest (in lieu of stress). As observed with stress, CORT treatment increased axon elongation and sprouting (p < 0.05 and p < 0.01 vs. Veh, respectively; Fig 10). Administration of dexamethasone (Dex; 2 mg/kg) was included to compare endogenous versus synthetic GC effects. Dex did not increase axon elongation but produced an intermediate effect on
axon sprouting (p < 0.05 vs. Veh; Fig. 10). These data indicate that stress enhances plasticity in a GC-dependent manner, presumably via activation of GR. To confirm this hypothesis, we are testing if a GR antagonist, RU486, prior to stress will abrogate stress effects on axon growth. Mice will be treated with RU486 prior to stress or no stress.

Figure 10. Corticosterone administration prior to DRG harvest enhances sensory axon growth. To determine if stress effects on growth are reproduced by treatment with corticosterone, mice were treated with CORT, in lieu of stress, prior to nerve injury. CORT treatment increased axon elongation (left; p < 0.05 vs. Veh) and sprouting (right; p < 0.01 vs. Veh). Treatment with the synthetic glucocorticoid, dexamethasone (Dex) increased axon sprouting (p < 0.05 vs. Veh). Results are expressed as mean ± SEM. *p<0.05, **p<0.01
Stress increases sensory neuron plasticity by NMDA receptor activation

Biochemical (e.g., ERK activation) and functional (e.g., LTP) neuron plasticity are downstream of NMDA receptor activation (Kim et al., 1996). Structural neuron changes, including spine growth and density dynamics (Ultanir et al., 2007), as well as axon growth (George et al., 2009; Lee et al., 2005; Schmitz et al., 2009) are regulated by NMDA receptors. Acute stress or GCs enhance glutamatergic signaling through NMDA receptors (Ahmed et al., 2006; Kim et al., 1996; Norris and Strickland, 2007; Wang et al., 2005b; Yang et al., 2008). To determine if axon plasticity is downstream of stress-induced NMDA receptor activation, mice were treated with memantine (MEM), an activity-dependent NMDA receptor antagonist, prior to stress. Axon growth was reduced by MEM pre-treatment (p < 0.0001 vs. vehicle + stress; Fig. 11) without interrupting typical axon plasticity (p > 0.05 vs. MEM + no stress; not shown). These data indicate that acute stress utilizes NMDA receptor signaling to drive sensory axon plasticity.
Stress activates NMDAR to enhance sensory neuron plasticity. To determine if stress effects on axon growth are mediated by NMDA receptor activation, mice were treated with memantine prior to stress. Memantine treatment prevented stress-induced axon growth at 96 h. Results are expressed as mean ± SEM. ***p<0.0001

**Stress-induced neuron plasticity is dependent on mTOR**

Activation of neuronal PI3K-Akt-mTOR represents a major axon growth signaling pathway (Park et al., 2008). To determine if stress-induced activation of mTOR signaling underlies increased axon growth, we tested the inhibition of axon growth by pre-stress treatment with rapamycin, an mTOR inhibitor. Pre-stress rapamycin robustly inhibited stress-induced axon growth (p < 0.001 vs. Veh + Stress) without affecting the growth from DRG neurons derived from non-stressed mice (p > 0.05 vs. Veh + No Stress; Fig. 12). Whereas mTOR signaling is normally suppressed after axon injury (Park et al., 2008), these data suggest that stress activates mTOR to increase axon growth.
Figure 12. Stress activates mTOR to increase axon growth. To determine if stress activates mTOR signaling to induce increased growth, we tested the inhibition of axon growth by rapamycin, an mTOR inhibitor, when administered prior to stress. Pre-stress rapamycin robustly inhibited stress-induced axon growth (p < 0.001 vs. Veh + Stress; denoted by ‘#’) without affecting the growth from DRG neurons derived from non-stressed mice (p > 0.05 vs. Veh + No Stress). Vehicle-treated stressed mice show increased axon growth versus no stress (p < 0.01; denoted by ‘**’). Scale bar = 40 μm.

Stress increases axon regeneration and pain-like behavior

Sensory neuron plasticity after injury might support axon regeneration. Stress-induced GC effects on intracellular signaling pathways associated with protein synthesis (e.g., mTOR) could enhance axon regeneration. To determine if stress conditions axon regeneration, regeneration after sciatic crush was measured. Sciatic nerve crush lesions were performed in mice immediately after 60 min restraint stress. Axon growth through the crush lesion was measured 9 days post-crush. The proportional area of SPRR1A expression, that is limited to regenerating axons, was determined as a measure of regeneration. SPRR1A expression in and beyond the lesion was increased by two fold in stressed mice (p < 0.05 vs. No Stress; Fig. 13A-C), indicating enhanced axon regeneration by stress.
Sensory axon plasticity is associated with the development of pain after injury. To determine if regenerative effects of stress are associated with increased neuropathic pain-like behaviors typically produced by nerve crush, mechanical thresholds to von Frey hair stimulation were measured on days 1, 3, and 7 post-crush. Heat sensitivity was assessed on days 2 and 8 post-crush. Mechanical allodynia was increased in stressed mice (p <0.05 vs. No Stress; Fig. 13D), whereas stress produced no effect on heat hyperalgesia after sciatic crush injury (Fig. 13E).
Figure 13. Stress improves axon regeneration but increases pain-like behavior after sciatic crush injury. To determine if stress increases axon regeneration in vivo, 60 min restraint was applied prior to sciatic nerve crush. A-C) On post-injury day 9, nerves were analyzed for the regeneration protein, SPRR1a. A & B) Representative images from no stress and stress groups, respectively. C) Stress increased SPRR1a expression in and below the crush site (p < 0.0001 vs. no stress). D) Stress increased allodynia after nerve injury (p < 0.05 on day 7 vs. no stress). E) Stress did not affect heat sensitivity after injury.
Discussion

In this study, we have identified acute stress as a determinant of sensory axon plasticity. DRG neurons derived from acutely stressed mice exhibit increased measures of axon sprouting and elongation. Likewise, corticosterone administration prior to DRG harvest also increases axon growth *ex vivo*. Stress and corticosterone effects on brain neurons are well-described; however, to our knowledge, this is the first example of stress altering sensory neuron plasticity. Indeed, the density of glucocorticoid receptor in DRG is impressively high, and dorsal spinal cord GR expression is comparable to hippocampus, suggesting an unappreciated role for stress and GC signaling in sensory pathways.

Stress studies in brain are common and provide multiple putative signaling pathways by which acute stress and GCs induce sensory neuron plasticity. Two predominant molecular programs affected by stress/GCs are MAPK (e.g., ERK1/2, p38, JNK) and mTOR (Ahmed et al., 2006; Norris and Strickland, 2007; Revest et al., 2005; Sasaguri et al., 2005; Shen et al., 2004; Shimizu et al., 2004; Yang et al., 2008; Zheng et al., 2007). In dorsal spinal cord, stress prior to nerve injury increases ERK 1/2 activation (Alexander et al., 2009). Both ERK and PI3K pathways are activated by NMDA receptors (Yang et al., 2008). Acute stress has previously been shown to activate mTOR in rat brain (Yang et al., 2008). Furthermore, ERK activation by stress could converge on the PI3K pathway to activate mTOR (Salmond et al., 2009).
Previously, we reported that stress immediately prior to peripheral nerve injury exacerbates neuropathic pain-like behavior. Elevated p-ERK in apparent dorsal horn neuron profiles indicated a potential role for stress in neuron plasticity (Alexander et al., 2009). Changes in ERK signaling reflect biochemical activity that could lead to multiple outcomes associated with pain. Here, using the same stress paradigm, we observe a form of sensory neuron plasticity, structural plasticity, to be affected. In the ex vivo preparation, as well as in vivo, in a sciatic nerve regeneration model, stress enhances axon growth. Ex vivo enhancement of axon sprouting and elongation are prevented by in vivo treatment with an open-channel NMDAR antagonist prior to stress, indicating that stress likely increases NMDAR activity to produce the observed axon plasticity. An important axon growth pathway that is downstream of stress-induced ERK and NMDAR activation is mTOR (Yang et al., 2008). If stress prior to DRG harvest or nerve crush activates mTOR to increase protein synthesis, increased axon growth, as we observed, would be expected. Indeed, inhibition of mTOR by rapamycin prior to restraint stress prevented stress-induced axon growth ex vivo.

In several brain regions, stress/GCs exert context-dependent effects on structural and functional neuron plasticity, as well as cognitive and emotional states. It is intriguing to consider that stress/GCs may produce similar dynamic control over sensory neurons and pain. If so, is axon plasticity inextricable from pain enhancement after stress? It may be difficult to dissociate the observed effect of stress on axon regeneration in vivo from pain using pharmacology, as virtually every molecule associated with functional plasticity
(e.g., enhanced neurotransmission) is redundant in its ability to enhance axon growth (e.g., NMDA, mTOR, ERK1/2, PI3K, BDNF). Indeed, functional plasticity associated with these molecules after neural injury is a hallmark of neuropathic pain.

With regard to pain, rapamycin delivery to the hind paw after nerve injury reduces pain-like behaviors (Jimenez-diaz, I 2008). Presumably, mTOR inhibition at this site decreased peripheral axonal sensitivity and perhaps concomitant processes of structural plasticity. Likewise, intrathecal administration of rapamycin is analgesic after nerve injury, with observed effects in the dorsal roots (peripherally) and in the dorsal horn (Geranton, sm, 2009). The utilization of the mTOR pathway by stress to increase sensory neuron activity may be dependent on functional neuron plasticity (e.g., LTP), as suggested by these studies. How stress activates mTOR signaling in sensory neurons is unknown. Acute stress activation of ERK and PI3K pathways may converge on mTOR and S6, as shown previously in hippocampus (Yang et al., 2008). Alternatively, stress may decrease inhibition of PI3K by PTEN.

In conclusion, this study demonstrates that acute stress prior to axon injury increases axon plasticity and regeneration. These data could be relevant in the development of pain or functional recovery after nerve injury. Dissociation of stress effects on pain and nerve regeneration would be desirable to achieve in such conditions as traumatic nerve injury, spinal cord injury, or diabetic neuropathy.
Chapter 4: Macrophage Migration Inhibitory Factor (MIF) is Essential for Persistent Inflammatory and Neuropathic Pain

Introduction

Elevated cytokine burden is common in persistent pain. Cytokines contribute to pain pathophysiology by generating or amplifying neuron sensitivity (Fukuoka et al., 1994; Jin and Gereau, 2006; Jung et al., 2008; Obreja et al., 2005; Oh et al., 2001; Ozaktay et al., 2006; Schafers et al., 2003a; von Banchet et al., 2005; Zhang et al., 2002). Accordingly, treatments that reduce pro-inflammatory cytokine activity are often analgesic (Lindenlaub et al., 2000; Sommer et al., 2001a; Sweitzer et al., 2001a). Macrophage migration inhibitory factor (MIF) is a unique cytokine that has not previously been studied in conditions of persistent pain.

Numerous cell types constitutively express MIF, including neurons (Bacher et al., 1998) and immune cells (macrophages, neutrophils, T cells and others). MIF exerts potent pro-inflammatory and chemotactic functions (Bernhagen et al., 2007; Calandra et al., 1998; Gregory et al., 2006) and is pathogenic in severe inflammation, atherosclerosis, and autoimmune diseases, including rheumatoid arthritis, colitis, and multiple sclerosis (Renner et al., 2005). It was the first discovered cytokine, yet its role in nervous system disorders, including neuropathic pain, is largely unknown (Bloom and Bennett, 1966; Calandra and Roger, 2003; David, 1966).
Unique among cytokines, MIF exhibits endocrine hormone activity and circadian regulation (Bernhagen et al., 1993; Petrovsky et al., 2003). Circulating MIF is ~1000 fold higher than other cytokine concentrations (e.g., IL-1b, TNF-a, IL-6) (Aloisi et al., 2005; Bucala, 1996). Unlike other cytokines, its release is stimulated by glucocorticoids (GCs) and stress, and it overrides GC-inhibition of NF-kB activity (Calandra et al., 1995; Daun and Cannon, 2000; Fingerle-Rowson et al., 2003), thereby promoting the production of pro-inflammatory cytokines.

Stress or GCs present at injury exacerbate neuropathic pain (Alexander et al., 2009; Norman et al., 2010). This observation might be attributable to the actions of MIF. Here, we show the first evidence that MIF is required for persistent inflammatory and neuropathic pain. Further, MIF appears to act as a downstream mediator of GC effects on pain; MIF inhibition prevents stress-induced exacerbation of pain. MIF may play a dual-role in pathology by increasing the pro-inflammatory potential of microglia and sensitizing neurons directly.

**Methods**

**Animals**

Adult female (8-10 weeks) MIF -/- mice generated as previously described (Bozza et al., 1999) were backcrossed for more than 10 generations to a C57Bl/6 genetic background. Wild-type (WT) mice of the same strain (C57Bl/6; Taconic, Germantown, NY, USA or
in-house colony) were used. All mice were group-housed in standard cages with *ad libitum* access to food and water. Mice were maintained in a vivarium with controlled temperature (~20°C) on a 12 hr light/dark cycle and were randomly assigned to experimental groups after a week habituation period. Behavioral testing was performed during the light cycle by an experimenter unaware of treatment. All procedures were conducted in accordance with protocols approved by the Ohio State University Institutional Laboratory Animal Care and Use Committee and with the guidelines of the Committee for Research and Ethical Issues of IASP.

**Drugs**

Recombinant MIF (rMIF) was obtained from xxx and delivered in sterile PBS vehicle. We tested the efficacy of two small molecule MIF inhibitors, Iso-1 (0.5-1 mg/mouse; Calbiochem (Gibbstown, NJ)) and MIFi (10 mg/kg; Cytokine Pharmasciences (King of Prussia, PA)). Both were prepared in 10% DMSO in sterile PBS. Lipopolysaccharide (LPS; E. coli 055:B5) and complete Freund’s adjuvant (CFA) were obtained from Sigma. Recombinant tumor necrosis factor-α (rTNF-α) was obtained from eBioscience (San Diego, CA, USA).

**Intraplantar inflammation**

A single subcutaneous (s.c) plantar right hind paw injection (0.02 ml) of complete Freund's adjuvant (CFA; 0.5 mg/ml suspension of heat-killed Mycobacterium tuberculosis in mineral oil; Sigma, St. Louis, MO, USA) was performed using a 26 gauge
sterile needle and syringe under brief anesthesia with 3% isoflurane in oxygen. Control mice were injected with the same volume of mineral oil, the vehicle for CFA and anesthetized as described above. Mice were housed on soft bedding. To assess the development of inflammation, hind paw volume was measured by plethysmometry immediately prior to adjuvant injection (baseline) and on days 1, 3, and 7 post-injection. This method of injection does not cause footdrop or autotomy, and the mice groom normally and appear healthy (Bortalanza et al., 2002).

Peripheral nerve injury

The spared nerve injury (SNI) procedure was performed as described previously by Shields et al. (Shields et al., 2003). Under isoflurane anesthesia (4% induction, 1.5% maintenance) in oxygen-enriched air and following shaving and aseptic preparation, an incision of the skin and biceps femoris muscle was introduced to expose the sciatic nerve and its three terminal branches at the upper-thigh level. Two of the branches, the sural and common peroneal nerves, were tightly ligated with 7-0 silk suture (Genzyme Biosurgery, Fall River, MA) and transected distal to the ligature. Subsequently, 1-2 mm of each nerve was resected, approximately 1 mm distal to the ligature. The tibial nerve was not disturbed. The muscle/fascia layer and skin layer were closed separately with 5-0 nylon suture (Syneture, Norwalk, CT).

Behavioral Testing
Punctate mechanical sensitivity was analyzed by measuring threshold response to von Frey filaments (Stoelting Co., Wood Dale, IL). Monofilament stimulation was applied to the mid-line of the plantar surface of each hind paw using the up-down method for threshold sensitivity (Chaplan et al., 1994). The stimulus intensity threshold represents the smallest force that elicited repeated withdrawal of the hind paw during 10 trials (≥ 50% response sensitivity to smallest force). Threshold values represent von Frey hair handle markings that correspond to log_{10} of (10x force in mg), as displayed in (Chacur et al., 2001).

Thermal sensitivity was tested using the Hargreaves’ method (Hargreaves et al., 1988) using the Plantar Test Apparatus (Ugo Basile, Comerio, Italy), as described previously (Hoschouer et al., 2010). The latency to withdraw the hind paw from the infrared radiant heat stimulus (25 W) is recorded. Both hind paws were tested for five trials in a randomized order with at least one minute between trials. To avoid tissue damage, the heat stimulus is disengaged after 30 sec for any non-responsive mice. The average duration across trials was used for data analyses.

Mice were acclimated to the testing procedure for 20 min each day for two days prior to the start of the experiment and for 10 min (von Frey) or 40 min (Hargreaves’) prior to the onset of each session. von Frey and Hargreaves’ measurements were conducted on different days to avoid overstimulation of the hind paws. All behavioral measurements were conducted by an investigator that was unaware of the treatment groups.
**In vitro microglia experiments**

The immortalized BV-2 microglia cell line was used to examine the effect of MIF on pro-inflammatory mediator production. Cells were treated with rMIF (10-100 ug/ml or Veh) for 6 h (gene expression) or 24 h (protein) at 37°C in a 5% CO₂ humidified incubator. The Veh control for MIF was sterile PBS in cell culture media (10% fetal bovine serum (Hyclone), 5% penicillin/streptamycin and 5% glutamax in DMEM (Gibco/Invitrogen). RNA was isolated using the Trizol method (Invitrogen) and cytokine transcript was measured using qRT-PCR as described below. Cellular protein was extracted using M-PER plus phosphatase and protease inhibitors, according to the manufacturer’s guidelines (Invitrogen).

**Quantitative RT-PCR (qRT-PCR)**

To determine MIF effects on microglial reactivity, select cytokines were analyzed from mRNA isolated from BV-2 cells. Gene-specific primer pairs for IL-1β, IkBa and iNOS were used as described previously (Kigerl et al., 2007). Gene expression was determined using qRT-PCR and compared between MIF and Veh groups. Primer sequence specificity was confirmed by performing blast analysis for highly similar sequences against known sequence databases. PCR reactions were carried out in triplicate using 1μL cDNA/reaction and SYBR Green master mix (Applied Biosystems, Foster City, CA, USA) in 20 μL reactions. PCR product was measured using SYBR Green fluorescence collected on an Applied Biosystems 7300 system (Ririe et al., 1997). Standard curves
were generated for each gene using a control cDNA dilution series. Melting point analyses were performed for each reaction to confirm single amplified products. ΔΔCt analysis was used to normalize gene data to 18s ribosomal RNA expression.

Flow cytometry

BV-2 cells were treated with activator cocktail and brefeldin A 4 h prior to collection. Cells were collected, washed and stained for CD11b (PacBlue), IL-6 (PE) and CCL2 (PerCP). Cells were analyzed using a FACSCanto II flow cytometer and FACSDiva software (BD Biosciences). Cells were gated based on forward vs. side scatter and CD11b expression. IL-6 and CCL2 were analyzed from the CD11b+ gate. Isotype control mAbs (Pharminogen) were matched for fluorochrome and used for cursor placement.

In vitro DRG experiments

Mouse DRGs were harvested as described previously (Gensel et al., 2009). Briefly, single cell suspensions of cervical, thoracic and lumbar DRG neurons were prepared from anesthetized adult (2-3 mo) mice. Dissected DRGs were incubated in dispase 2 (5 U/ml; Roche, Penzbeg, Germany) and collagenase type 2 (200U/ml; Worthington, Lakewood, NJ) for 45-60 min at 37°C in Hanks' Balanced Salt Solution (HBSS; Mediatech, Herndon, VA), followed by DNase 1 type 2 (250 µg/ml; Sigma) treatment for 5 min. Next, DRGs were triturated in 500 µl HBSS media through Pasteur pipettes and spun at 3000 rpm for 3 min. The neuron-enriched pellet was re-suspended in 100 µl of Neurobasal A media supplemented with 2% B27, 1% Glutamax™, and 1% penicillin-105
streptomycin. All media components were obtained from Invitrogen (Gland Island, NY). Neurons were plated onto coverslips at 400 cells/coverslip or 10K/coverslip for high-density growth analysis. All coverslips were pre-coated with 0.1 mg/ml of poly-L-lysine (Sigma) and 10 µg/ml of laminin (Invitrogen). After 24-72 h at 37ºC in a 5% CO₂ humidified incubator, cells were utilized for electrophysiology (24 h) or measurements of axon growth (72 h).

**Electrophysiology**

Plated coverslips were centered in a perfusion chamber filled with extracellular solution (ECS) containing (in mM): 145 NaCl, 5 KCl, 1 MgCl₂, 2 CaCl₂, 10 Glucose, 10 HEPES, pH7.4. rMIF was diluted in ECS and delivered via a pressure-driven perfusion system (SmartSquirt 8, AutoMate Scientific) with the tip positioned so that the DRG neuron was within the direct stream of perfusate. Recording pipettes were pulled from micropipette glass (A-M Systems, Inc., Carlsborg, WA) to 3-5 MΩ and filled with an intracellular solution containing (in mM): 122 K-gluconate, 9 NaCl, 1.8 MgCl₂, 0.9 EGTA, 9 HEPES, 14 Tris-creatinePO₄, 4 MgATP, 0.3 Tirs-GTP, pH 7.2). Tight (GΩ) seal and high quality break-in were made by utilizing the ez-gSEAL pressure controller (NeoBiosystems, Inc. San Jose, CA). Whole cell recordings were accomplished using an EPC10 amplifier and the PatchMaster 2.2.0 software (HEKA Electronik, Germany). As soon as the whole cell configuration was established, fast and slow capacitances were cancelled and the holding potential (Vh) was set to -60 mV. Resting membrane potential (RMP) and spontaneous firing (SF) were recorded after switching to current clamp mode, and setting current
injection to zero. Depolarization-induced firing was established by injecting a sufficient current to maintain resting membrane potential at -30 mV in current clamp mode. Data were filtered at 3 kHz and digitized at 10 kHz. All recordings were made at room temperature (22-24 °C). Only one cell per cover slip was recorded to avoid possible drug contamination of other cells.

ECS perfusion was initiated as soon as coverslip was set in the recording chamber. The neuron that was being recorded was perfused continuously either by ECS or 1 ng/ml rMIF in ECS. SF or depolarizing-induced firing was recorded for 12 seconds each, before, during (1 min after initiating rMIF perfusion) and after rMIF treatment.

Data analysis

Behavioral data were analyzed using repeated measures ANOVA. Post-hoc analyses were conducted using Bonferroni’s test. Factorial ANOVA was used for histological analyses. Student’s t test was used to analyze behavioral data for individual time points and between-group gene expression analyses. An α-level of p < 0.05 was used as an indication of statistical significance.
Results

MIF contributes to neuropathic pain-like behavior

Constitutive expression of MIF has been identified in most cells. Relevant to pain, MIF has been observed in rat DRG, sciatic nerve, spinal cord, and brain (Nishio et al., 1999; Vera and Meyer-Siegler, 2003; Wang et al., 2010). Nishio and colleagues show increased expression in rat sciatic nerve axons and glial cells after transection injury (Nishio et al., 1999). Using multiple commercially available antibodies and time points post-injury, we observed MIF immunoreactivity (IR) in mouse sciatic nerve, DRG, and spinal cord; however, without an apparent increase in MIF IR after SNI. Likewise, in rats using IHC, at early time points post-SNI (1-6 h) and shams, we observed comparable MIF in the lumbar spinal cord, DRG, and sciatic nerve. Perhaps another method of identifying MIF expression (e.g., Western blot) would demonstrate elevated MIF after SNI; alternatively, constitutive and widespread expression of MIF may be unchanged in this model of sciatic nerve injury.

MIF contributes to neuropathic pain after peripheral nervous system injury

In persistent inflammatory and neuropathic pain, it is common for normally innocuous stimuli to produce pain; this characteristic is termed ‘alldynia.’ To determine if MIF plays a role in neuropathic pain, alldynia was assessed in WT and MIF KO mice. After nerve injury, WT mice develop robust sensitivity to low-force mechanical stimulation (p < 0.0001) that returns to baseline levels by 28 d. In contrast, MIF KO mice do not
develop allodynia throughout the duration of the study (p > 0.05, and p < 0.005 vs. WT; Fig 14A).

Stress and elevated GCs are, at times, coincident with the development of pain in humans, and both exacerbate allodynia after nerve injury in mice (Alexander et al., 2009). Because stress and GCs induce MIF, we tested the hypothesis that MIF contributes to stress-induced enhancement of allodynia after nerve injury in mice that lack MIF. As shown previously, nerve injury produces allodynia (p < 0.0001 vs. sham) that is enhanced by stress (p < 0.001 vs. no stress; Fig 14B). Again, MIF KO mice exhibit no change in mechanical threshold after nerve injury (p > 0.05 vs. sham; Fig 14A), and stress prior to nerve injury has no effect on KO mice (p > 0.05 vs. no stress; Fig 14A). These data indicate that MIF KO mice are protected from stress-induced exacerbation of allodynia.

Because the MIF KO mice do not develop pain-like behavior after nerve injury, regardless of stress, we examined the role of MIF in stress-induced enhancement of allodynia using a small molecule inhibitor of MIF. Nerve injury produced mechanical allodynia in all mice (p < 0.0001 vs. baseline; Fig 14C). Stress prior to nerve injury exacerbated allodynia in veh-treated mice (p < 0.01 vs. no stress) that was prevented by pre-stress treatment with the MIF inhibitor (p < 0.01 vs. veh; Fig 14C). These data indicate that stress-induced enhancement of pain-like behavior after nerve injury is dependent on MIF.
Finally, to determine if allodynia can be reduced by MIF inhibition after nerve injury, mice were treated with the MIF inhibitor, Iso-1 (1 mg/mouse), 30-60 min post-injury. Iso-1 (0.5 mg/mouse) was also administered on days 1-3 post-SNI. Allodynia was tested 2 h post Iso-1 treatment on days 1 and 3 post-SNI; to determine the duration of analgesia, mice were tested on days 7 and 14 (treatment was suspended on day 3). Iso-1 treatment attenuated allodynia (p < 0.0001 vs. veh; Fig 14D). These data were replicated in a subsequent study. The long-term reduction of allodynia by early treatment with Iso-1 indicates that MIF is important for the initiation of pain-like behavior.
Figure 14. MIF is critical for neuropathic pain-like behavior and stress-induced pain enhancement. A) MIF KO mice do not develop allodynia after nerve injury. B) Nerve injury produces allodynia in WT mice that is exacerbated by acute stress (60 min restraint). MIF KO mice do not develop allodynia, regardless of stress. C) A small molecule inhibitor of MIF (MIFi) prior to stress prevents stress-induced enhancement of allodynia after nerve injury. D) Treatment with another MIF inhibitor (Iso-1) attenuates allodynia after nerve injury. Results are expressed as mean ± SEM. N=5/group. Y-axis
values represent von Frey hair handle markings. #: WT No Stress vs. Stress; *: WT vs. KO. #/*p<0.05; ##/*p<0.01; ###/*p<0.0001.

MIF KO mice exhibit decreased pain-like responses in a model of inflammatory pain

To determine if MIF is a critical mediator of persistent pain in general, its role was examined in a model of chronic inflammatory pain. To determine if MIF is pro-algesic in inflammatory pain, MIF KO mice were injected i.pl. with CFA. MIF KO mice show no baseline deficit or increased sensitivity to mechanical and heat stimuli (p > 0.05 vs. WT mice; Fig 15A, B). Paw edema develops in both WT and MIF KO mice (p < 0.001 vs. baseline), but peak edema is less severe in MIF KO mice (p < 0.01 vs. WT mice; Fig 15C). After CFA, WT mice develop mechanical allodynia (p < 0.001) and thermal hyperalgesia (p < 0.05); however, MIF KO mice do not show increased sensitivity to mechanical (p > 0.05 vs. baseline and p < 0.001 vs. WT mice) or heat stimuli (p > 0.05 vs. baseline and p < 0.05 vs. WT; Fig 15D, E).
Figure 15. MIF KO mice do not develop inflammatory pain. MIF KO mice exhibit typical nociceptive behavior but resistance to CFA-induced inflammatory pain. Baseline mechanical (A) and thermal (B) thresholds are similar in MIF KO and WT mice (n=10-12/group). Peak edema after CFA is attenuated in MIF KO mice (C). Intraplantar CFA induces persistent mechanical allodynia (D) and thermal hyperalgesia (E) in WT mice that is absent in MIF KO mice (n=5/group). Results are expressed as mean ± SEM. PID: post-injection day. D: y-axis values represent von Frey hair handle markings. *p<0.05; **p<0.01; ***p<0.0001.

*MIF is an algogenic cytokine*

The absence of evoked pain behavior in MIF KO mice and reversal by a MIF inhibitor suggests that MIF is a critical regulator of pain. Next, we tested if rMIF is directly pro-
algesic. Indeed, intraplantar administration of rMIF (1000 pg) is sufficient to produce allodynia in uninjured WT mice (Fig 16A; p < 0.05 and p < 0.0001 vs. veh at 3 and 5 h, respectively). We compared rMIF-induced behavior to TNF-α (100 pg), a cytokine and dose previously shown to produce allodynia (Cunha et al., 2005; Jin and Gereau, 2006; Schafers et al., 2003b). As expected, TNF-α induced mechanical sensitivity (Fig 16A; p < 0.05 vs. veh at 3 h) and was comparable to rMIF effects (p > 0.05 vs. TNF-α). Other cytokines/chemokines shown to produce pain-like behavior require higher nanogram concentrations (e.g., CCL2, CXCL1, IL-6) (Abbadie et al., 2003; Qin et al., 2005), suggesting rMIF is a potent pro-algesic cytokine.

To determine if rMIF-induced allodynia is susceptible to stress effects, acute restraint stress was administered prior to i.pl. rMIF (1000 pg). As observed in the nerve injury model (Alexander et al., 2009), stress increased allodynia after rMIF treatment (Fig 16B; p < 0.0001 vs. no stress).
Figure 16. MIF is an algogenic cytokine. Intraplantar administration of rMIF (1000 pg) produced allodynia (p < 0.05 at 3 h and p < 0.001 at 5 h vs. saline) comparable to that elicited by TNF-α (A; 100 pg; p > 0.05 vs. rMIF). #: TNF-α vs. saline; *: rMIF vs. saline. Acute stress (60 min restraint) prior to rMIF (1000 pg) administration increased allodynia (B; vs. no stress). N=5/group. #/*p<0.05; **p<0.01; ***p<0.0001

*MIF induces a pro-inflammatory phenotype in microglia*

The expression of CXCR2/CD74 on neurons and microglia indicates that MIF may produce or exaggerate pain by acting on either cell type. Although MIF effects have been demonstrated for numerous immune cells, microglia have not been examined. To test the capacity of MIF to induce a pro-inflammatory microglia phenotype, primary rat microglia were treated with rMIF (10, 30, 100 ng/ml) *in vitro* (Fig 17). Transcript levels of iNOS, IκBα, IL-1β, CCL2, and TNF-α were elevated after 6 h of rMIF treatment in three
independent replicate experiments (Fig. 17 A-E). A subset of these cytokines were tested and confirmed using mouse primary microglia and BV-2 immortalized microglial cells (data not shown).
Figure 17. MIF induces a pro-inflammatory phenotype in microglia. Treatment of rat primary microglia with rMIF for 6 h increases transcript levels of iNOS (A), IkBα, an indicator of NFκB activation (B), IL-1β (C), CCL2 (D), and TNF-α (E). Data are normalized to the internal control gene (18s), and expressed relative to media control.

Using flow cytometry, we quantified IL-6 and CCL2 in BV-2 microglial cells after rMIF treatment (Fig. 18 and 19). Treatment with 100 ng/ml rMIF for short duration (12 h) robustly increases intracellular CCL2 and IL-6, whereas lower concentrations (10, 30 ng/ml) are without effect (Fig. 18). In contrast, longer duration (24 h) rMIF treatment
produces elevated intracellular CCL2 and IL-6 only in the 10 ng/ml concentration (Fig. 19). The potential down-regulation of CCL2 and IL-6 in the 30 and 100 ng/ml treated wells at 24 h may indicate earlier accumulation and secretion of these higher concentration rMIF treatments. Replicate experiments are needed to confirm these observations.

Figure 18. Intracellular CCL2 and IL-6 protein in BV-2 microglia after 12 h rMIF treatment. CCL2 and IL-6 expression were measured by gating on CD11b+ events. Representative contour plots show A) CCL2 and B) IL-6 expression profiles in CD11b+ BV-2 microglia after 12 h of media or rMIF treatment. Individual gates are labeled with the mean percent of CCL2 or IL-6 expressing CD11b+ cells for each group.
Figure 19. Intracellular CCL2 and IL-6 protein in BV-2 microglia after 24 h rMIF treatment. CCL2 and IL-6 expression were measured by gating on CD11b+ events. Representative contour plots show A) CCL2 and B) IL-6 expression profiles in CD11b+ BV-2 microglia after 24 h of media or rMIF treatment. Individual gates are labeled with the mean percent of CCL2 or IL-6 expressing CD11b+ cells for each group.

**MIF induces plasticity and functional activation of sensory neurons**

CXCR2/CD74 expression by neurons suggests neurons are responsive to MIF. To test this, sensory neurons were treated with MIF *ex vivo*. Axon sprouting is a measure of neuron plasticity that is associated with the development of pain after injury. rMIF (100 and 1000 pg/ml) increased measures of axon sprouting by sensory neurons (p < 0.01 vs. veh for both concentrations; Fig 20C). rMIF (1000 pg/ml) also increased axon length (p <
0.01 vs. veh; Fig 20B). Because sprouting measurements are affected by axon length, axon sprouting was measured within 500 um of the soma; here, the sprouting effect of rMIF treatment was still evident (100 pg/ml: p < 0.01 vs. veh; 1000 pg/ml: p < 0.05 vs. veh; Fig 20D). Next, we compared rMIF induced growth to a well-established growth factor, nerve growth factor (10 ng/ml). NGF increased growth relative to veh (p < 0.05), but NGF was comparable to rMIF (1 ng/ml; p > 0.05; data not shown).
Figure 20. rMIF increases sensory neuron plasticity. DRG neuron cultures were treated at 24 h with rMIF (10, 100, 1000 pg/ml). At 72 h, neurite outgrowth length and branching complexity was examined by Sholl analysis. A composite of this plasticity is increased by rMIF (A; p < 0.01, only 1000 pg/ml shown). rMIF treatment (1000 pg/ml) increased long-distance axon growth (B) and sprouting/branching complexity (C,D). Representative neurons demonstrating mean axon length in Vehicle (E) and rMIF (F) treated cultures. The sprouting phenotype was measured across the entire neuron (C) and then within 500 μm of the soma (D) to discount the contribution of increased length in rMIF-treated cells. Results are expressed as mean ± SEM. N= ≥ 76 neurons per group (all neurons fitting criteria per coverslip (see Methods). Scale bar in D & H = 100μm. *p<0.05; **p<0.01.
Figure 20.

A) Area Under the Curve

B) Axon Length

C) Axon Sprouting

D) Axon Sprouting <500 µm
MIF treatment induces an excitatory effect on DRG neurons. Neurons that fire spontaneously during current clamp exhibit increasing response frequency during injection of rMIF (1 ng/ml; Fig 21). rMIF treatment appears to have an instantaneous effect specifically on this population of small diameter sensory neurons.

Figure 21. MIF increases the excitability of spontaneously active small diameter DRG neurons. Current-clamp recordings before (A) and after rMIF (B; 1 ng/ml) from the same neuron. C) Mean neuron spike frequency measured every 12 s after current clamp at -30 mV and after 1.5-3 min rMIF exposure (n=7). *p < 0.05

Discussion

The present data show a critical role for MIF in inflammatory and neuropathic pain. Mice lacking MIF were protected from pain-like behavior after CFA and nerve injury. Furthermore, an inhibitor of MIF reversed established allodynia after nerve injury. MIF may be directly pro-algesic, as rMIF alone produced allodynia. Despite its established
inflammatory functions and presence in relevant cell types, the role of MIF in pain conditions has only recently been tested (Wang et al., 2010). In a formalin model of acute inflammatory pain, MIF inhibition reduced pain-like behavior in rats (Wang et al., 2010). MIF’s newly appreciated role in acute pain and here, in persistent pain is an important discovery for the field of pain. The emergence of new roles for classical inflammatory cytokines and chemokines as neuron regulators is growing. MIF is perhaps the best example of a molecule that bridges the immune, endocrine, and nervous systems. Although it is now apparent that MIF plays an important role in persistent pain states, where MIF fits into our current understanding of pain pathophysiology is yet to be determined.

It is intriguing to consider that MIF may act as a fast responder to nervous system dysfunction, and may thereby initiate or propagate immune or neural sequelae. In support of this possibility, first, MIF is induced by elevated GCs (Calandra et al., 1995), a signature of disrupted homeostasis. Second, unlike most cytokines, it is constitutively expressed by virtually every cell enabling rapid release and wide distribution without need to synthesize protein (Bernhagen et al., 1993). Third, it is highly pro-inflammatory. Finally, as described herein, it is neuro-active. MIF receptors are present on neurons (Bryan et al., 2008) and rMIF treatment leads to increased measures of plasticity. Still, it remains to be tested if the typical cadre of cytokines is downstream of MIF. MIF regulation of PU.1, a transcription factor required for TLR4 gene expression (Roger et al., 2001), supports this notion.
At present, there is no explanation for variable susceptibility to neuropathic pain after precipitating conditions. It is possible that hormone status at the time of injury, or during the course of pathology, determines susceptibility to develop neuropathic pain. Among the putative determinants of susceptibility are glucocorticoids and the cytokine-hormone MIF. Indeed, clinically, stress increases susceptibility to develop pain and exacerbates existing pain (Ashkinazi and Vershinina, 1999; DeLeo, 2006; Greco et al., 2004; Nicholson and Martelli, 2004; Turner et al., 2002). Elevated stress hormones (e.g., GCs) at the time of injury exacerbate neuropathic pain in mice (Alexander et al., 2009). The induction of MIF by GCs/stress suggests a potential pathway whereby GCs/stress exacerbates pain. Like corticosterone, rat plasma MIF increases after exposure to stress (Calandra et al., 1995). From the present data, stress effects on pain depend on MIF activation. Pre-stress treatment with a MIF inhibitor prevented stress-induced enhancement of allodynia after nerve injury.

Elevation of MIF during stress, or after injury, could act on either the immune or nervous systems to affect pain. To our knowledge, we have shown for the first time a pro-inflammatory effect of MIF on microglia. Treatment of primary microglia increased transcript and protein levels of IL-1β and TNF-α, as well as iNOS and its product, nitric oxide. The upregulation of IkBa transcript indicates NFκB activation in microglia by rMIF. The secretion of these factors by immune cells not only supports inflammation, but
could act either directly or indirectly to facilitate peripheral and central neuron sensitization.

The role of MIF in neurons is unknown. Neurons express the MIF receptors, CD74 (Bryan et al., 2008), CXCR2 and CXCR4 (Hesselgesser et al., 1997; Meucci et al., 1998; Xia and Hyman, 2002). MIF can also enter some cells by non-receptor mediated endocytosis (Kleemann et al., 2002); whether this is possible in neurons is unknown. Notably, upregulation of the MIF receptor gene, CD74, has been observed in rat brain following awake LTP induction protocol (Havik et al., 2007) and an emotional learning task (Zhang et al., 2005b). The cellular localization of CD74 was not determined in these studies. A recent study shows that MIF KO mice exhibit increased anxiety and depressive behavior and impaired hippocampal-dependent learning and memory (Conboy et al., 2010). This behavioral phenotype is likely to result from neuron dysfunction, implying a role of MIF in normal neuron plasticity; however these effects could result from altered glial-neuronal communication. If MIF signaling in neurons resembles other cells, MIF will induce MAPK activation (Lue et al., 2005; Mitchell et al., 1999; Santos et al., 2004). ERK, in particular, is activated by MIF in numerous conditions; in neurons, this would support mechanisms of neuroplasticity, including axon growth and LTP. Here, we show that MIF treatment of sensory neurons increases neurite outgrowth. While long-distance axon growth could facilitate regeneration after injury, we also observed increased branching complexity that is typical of sprouting. Axon sprouting is associated with the development of pain and spasticity, supporting a role for MIF in neuropathic conditions.
The functional plasticity induced by rMIF on putative nociceptors would support central sensitization and pain.

MIF was the first discovered cytokine, and today it is well-described for its role in inflammatory diseases. Surprisingly, the role of MIF in nervous system diseases is largely unexplored. Indeed, most nervous system pathology is influenced by immune responses, and therefore a role for MIF will likely be elaborated in the future. We show that MIF is essential for persistent inflammatory and neuropathic pain, conditions that clearly incorporate neural and immune mechanisms.
Chapter 5: General Discussion

The quest for analgesia

The clinical problem of neuropathic pain is our present inability to provide adequate analgesic relief. Tens of millions of people worldwide suffer every day, often for many years (Bouhassira et al., 2008; Dieleman et al., 2008; Gilron et al., 2006; Taylor, 2006). Neuropathic pain can develop at any age, and in both sexes of any race. Pain is often concomitant with an antecedent condition, meaning that pain is not the only challenge patients and physicians confront. It is easy to imagine the impact of living with persistent pain. The physical, psychological, and financial burden can be devastating to self and family (Turk et al., 2008). Adding insult to injury, accumulating evidence indicates that chronic pain impairs cognitive function and shrinks brain volume (Apkarian et al., 2004a; Apkarian et al., 2004b; Baliki et al., 2008; Geha et al., 2008; Seminowicz et al., 2009). Pain sufferers are seeking help, and are generally unsatisfied with available measures (Baron and Tolle, 2008). To this end, scientists seek to resolve the mysteries that make pain relief so elusive.

The ‘mysteries’ of persistent pain pathophysiology, including neuropathic pain, are steadily being resolved. Understanding the mechanisms of these conditions should eventually translate into useful preventative and therapeutic strategies. An important aspect of neuropathic pain is its unpredictability. Not all individuals who experience a
precipitating condition (e.g., spinal cord injury, stroke, diabetes) develop neuropathic pain. There are no known biomarkers, and any genetic underpinnings are as yet not identified. This presents a challenge to physicians who wish to apply preventative measures during the course of relevant disease (e.g., spinal cord injury, cancer). One potential determinant of pain susceptibility is endocrine hormone status.

The endocrine system is uniquely situated to exert control over pain etiologies. First, many of its hormone constituents have unrestricted access to the entire body, including the nervous system. For example, steroid hormones are lipophilic and thereby readily diffuse into cells. Second, many hormones are pleiotropic, affecting numerous cell types in multiple capacities. Third, many hormones follow a circadian rhythm that reflects their significant regulatory role in physiology (or pathophysiology, in the case of dysfunction). Deviation from the rhythm occurs in response to disrupted homeostasis.

Glucocorticoids are pleiotropic steroid hormones that exhibit a circadian rhythm and respond to homeostatic challenges. Stressors are robust inducers of GC release; in the case of humans, the threat to homeostasis is often perceived, not real. For example, in modern, industrialized society, we more often stimulate GC release by ‘stressing’ over money or job performance than because of a physical threat to our survival, which is the intended adaptive function of the circuitry. Other inducers of GCs that more readily qualify as homeostatic challenges than psychological stress, include metabolic (e.g., exercise, fasting) and immune/systemic (injury, infection). It should be clear by now why hormones, particularly GCs, represent a prime factor for influencing pain pathology.
They are mobilized by injury, have wide access to tissues, and exert multifarious effects. To discount the role of GCs and, more broadly, the endocrine system in nervous system pathology would be akin to overlooking the influence of cytokines and the immune system. Incidentally, this was the case for immune system interest in pain research until about 1990! By this account, perhaps the role of the endocrine system in pain will gain attention over the next decade.

The merging of pain and endocrine stress hormones (e.g., GCs) research areas in my graduate research was a natural and highly valid combination. Inherent to the struggle of chronic pain is a certain amount of stress. In humans, stress increases susceptibility to develop pain and exacerbates existing pain (Ashkinazi & Vershinina, 1999; DeLeo, 2006; Greco et al., 2004; Nicholson & Martelli, 2004; Turner et al., 2002). Other pain comorbidities include the closely associated experiences of anxiety and depression (Nicholson and Verma, 2004). Despite a clear clinical relationship of pain and stress, there are surprisingly few basic research studies that address this problem. To our knowledge, we were the first to show that acute stress, or elevated glucocorticoids, prior to nerve injury exacerbate neuropathic pain-like behaviors (Alexander et al., 2009). We (and others) have yet to examine the impact of chronic stress on established neuropathic pain, which we appreciate is highly relevant to the human experience.

When studying the role of stress in any context, it is important to consider the timing, duration, and intensity of the stressor. For GC administration, the timing, duration, dose,
and specific compound are important determinants of outcome. I predict that, for the above scenario of established pain, a chronic stressor will enhance pain. Acute pain in this context, when the pathology is already *underway*, is unlikely to produce a sustained difference in pain behavior. Indeed, a pilot study with such a protocol revealed that acute restraint stress one day post-nerve injury had no effect when evaluated one day later (unpublished data). Having observed a robust effect of acute stress prior to injury on pain, my objective has been to delineate the mechanisms of this process, rather than undertake a systematic series of studies describing the stress-pain dynamic. In the future, I would like to explore such studies as I think the outcomes will shed light on the specifics of a stress hormone-pain relationship. It is worth mentioning here that our colleagues, Norman et al., observed a pain enhancing effect of chronic stress prior to nerve injury (Norman et al., 2010). Their study, like ours, exposed mice to restraint stress immediately prior to nerve injury; therefore it is unclear if chronic stress is necessary to exacerbate pain, or rather if acute is sufficient. Together with our studies, it is apparent that elevated glucocorticoids at the time of injury alter the subsequent pathology in such a way to increase pain-like behavior.

_Mechanisms of stress-induced exacerbation of pain_

Acute restraint stress or elevated GCs prior to nerve injury enhances pain-like behavior, but how? In our studies, we attempted to localize the GC effect to cellular and molecular targets. Considering the multiplicity of known contributors to persistent pain (see Table 5), the task of identifying how stress impacts pain is not easily accomplished. A logical
approach was to consider stress effects in substrates that overlap with pathways pertinent to pain pathology. For example, microglia are putative effector cells because stress has previously been shown to induce microglial reactivity (Sugama et al., 2007; Nair and Bonneau, 2007), and microglial responses to nerve injury are pivotal to the development of pain behavior (Jin et al., 2003; Narita et al., 2006; Tsuda et al., 2003, 2004). We reasoned that stress might prime the pro-inflammatory phenotype of microglia to nerve injury, and thereby potentiate their typical response, ultimately facilitating pain. To simplify this section, I have divided it into neural and immune mechanisms of stress effects on pain. This is over-simplified, as I appreciate that there is substantial cross-over between effectors of these systems; however, the designations are based on classical functions of the systems and their constituents.

Neural mechanisms of stress-induced exacerbation of pain

As described above, stress and GCs may exacerbate neuropathic pain through modulation of neuroplasticity (Blackburn-Munro and Blackburn-Munro, 2003; Joels and Krugers, 2007; Revest et al., 2005; Wang et al., 2005b). We have conceptualized neuron plasticity in terms of function (e.g., LTP, LTD), structure (e.g., axon regeneration, sprouting, reorganization; synapse formation), and biochemistry (e.g., p-ERK, PI3K). Our present experiments test each of these qualities, though much remains to be learned in regards to stress-induced enhancement of allodynia. In hippocampus and amygdala, stress and GCs facilitate long-term potentiation (LTP) or depression (LTD), depending on the type and duration of stressor (Ahmed et al., 2006; Coussens et al., 1997; Diamond et al., 1992;
Kim et al., 1996; Pavlides et al., 1993). Acute stress induces hippocampal LTP using the same machinery we appreciate in neuropathic pain signaling (e.g., increased activated forms of ERK1/2, p38, CaMKII, and CREB (Ahmed et al., 2006). Would acute stress, then, combined with neural injury facilitate functional mechanisms of neuropathic pain? This remains to be tested in future studies. Perhaps first we would consider using in vitro dorsal horn neuron preparation combined with DRG neurons derived from stressed mice to evaluate synapse formation, strength, and transmitter release.

In comparison, structural plasticity has received less attention in neuropathic pain in the last decade. Some limitations of tracing procedures drew controversy to the observations that neurons sprout and reorganize after peripheral nerve injury (Woolf 1992, 1995). With advancements in transgenic mice, these techniques could now be combined to demonstrate the contribution of structural plasticity to pain. In other areas of the nervous system, acute stress (or GCs) induces biochemical and structural neuron plasticity (Mitra and Sapolsky, 2008; Pawlak et al., 2003). Whether this phenomenon occurred in peripheral sensory neurons was unknown. Indeed, in DRG neurons, we observed that stress prior to axon injury increases axon sprouting and elongation.

*Immune mechanisms of stress-induced exacerbation of pain*

In most tissue sites, the innate immune response fulfills its purpose; it responds to and defends against infection, prevents damage and initiates repair of injured or infected tissue (Yeager et al., 2004). The return to homeostasis following nervous system damage,
however, is constrained. It appears that the chronic, feed-forward nature of neuropathic pain reflects unresolved inflammation and concomitant neuronal sensitization. The majority of inflammatory mediators are algogenic, and further sensitize pain transmission pathways by producing ectopic firing at the injury site and sensitization in the CNS. In this regard, the immune response to nervous system injury is mechanistic in the generation and maintenance of pain.

As mentioned above, microglia are a good place to start in a discussion of neuroinflammatory and immune mechanisms. Microglia have dominated research in this field. Although the field is now shifting away from it, in the past, microglial morphology changes were widely accepted as informative. An increasing number of studies report alterations of pain behavior without diminution of microglial reactivity (Colburn et al., 1997; Tozaki-Saitoh et al., 2008; Tsuda et al., 2009b; Tsuda et al., 2003); thus, morphology and expression of cell surface markers alone is an inadequate measure of microglial contribution to neuropathic pain-like behavior. The term ‘neuroinflammation’ should be distinguished from resident glial reactivity (e.g., microglia and astrocytes) that occurs in response to a range of stimuli. We observed a small effect of acute stress on early (POD 1) microglial morphology, but later (POD 8) this effect was absent despite a persistent behavioral effect (Chapter 2). In subsequent experiments, based on behavior and MIF biology, we would have predicted reduced measures of microglial reactivity in MIF KO mice; in contrast, however, Iba-1 IR was increased in MIF KO mice (data not shown). It is possible, that in certain circumstances, microglial reactivity (as measured
by morphology and common surface markers) is either insignificant, or perhaps even protective/analgesic.

Intracellular signaling in microglia is a more consistent determinant of changes in pain behavior than morphology and cell surface markers. There is a growing list of critical molecules for pain-like behavior in rodent models of persistent pain. How to reconcile the observation that deletion or inhibition of a single factor blocks pain behavior in approximately one-half dozen distinct cases is difficult, except to assume that perhaps there is a chronological succession or interaction among these distinct molecules. At present, gene knockouts for PKCγ, TLR2, TLR4, CCR2, IFN-γ, P2X4R, MMP9, and now MIF show protection from neuropathic pain-like behavior after nerve injury (Abbadie et al., 2003; Kawasaki et al., 2008; Kim et al., 2007; Tanga et al., 2005; Tsuda et al., 2009a; Tsuda et al., 2009b; Zhang et al., 2007). Based on MIF’s unique constitutive availability, we predict it could be a possible upstream determinant of other inflammatory mediators. This concept remains to be tested in future experiments. Downstream of these microglial/macrophage mediators are several kinases that are also pivotal to neuropathic pain-like behavior, including p38, ERK, and Src (Garry et al., 2005; Inoue et al., 2003; Ji et al., 1999; Jin et al., 2003; Katsura et al., 2006; Obata et al., 2004; Tsuda et al., 2004; Tsuda et al., 2008; Zhang et al., 2005a). A role for acute stress in activation of these kinases has been demonstrated in discrete research areas, as described above. The timing of stress-induced kinase activity could modulate subsequent
neural responses to injury in such a way to propel the inflammatory and neuronal mechanisms of neuropathic pain.

*Significance and Conclusions*

Neuropathic pain is clearly a devastating condition. It robs people by the millions of a normal life. Stress is an unavoidable aspect of everyday living, but it too can strongly interfere with wellbeing. In pain conditions, stress is said to be an aggravating factor. This stress-pain relationship is documented clinically, but without explanation. Thus, the onus is on clinicians and basic scientists to describe its mechanism. Surprisingly, there is a dearth of literature in this regard. To our knowledge, we were the first to test how stress affects the development of neuropathic pain. How chronic stress alters the course and severity of established neuropathic pain remains to be tested in future studies.

Acute elevation of stress hormones prior to neural injury, as we have studied, demonstrates remarkable effects on the severity of neuropathic pain-like behavior. As described in Chapter 2, 60 minutes of restraint stress immediately before peripheral nerve injury potentiates subsequent allodynia in mice. This is an important observation for those who suffer from conditions that lead to neuropathic pain (e.g., stroke, SCI), as well as those with episodic pain. It suggests that stress could impact the onset or severity of pain. Moreover, although the experimental procedure leads to pain-like behaviors in all mice of this strain, not all humans develop neuropathic pain after a precipitating condition. Perhaps our observation, when translated to the human condition, will instead
reveal that elevated stress hormones increase the susceptibility to develop pain. If so, this would have an enormous impact on preventative care; at present, there is no way to predict and thus prevent the development of neuropathic pain.

When the receptor (GR) for the primary stress hormone, corticosterone, was pharmacologically inhibited, stress effects on alldynia were mitigated. Like the stressor, systemic administration of corticosterone prior to nerve injury enhanced pain-like behavior in mice (Chapter 2). These data implicate glucocorticoids as a mediator of the observed stress effect. This information may be helpful to the human condition; however, long-term blockade of this pleiotropic, widespread receptor function will likely produce undesirable effects. Indeed, our data often surprise those who are only familiar with the dogmatic role of GCs as counter-inflammatory and immunosuppressant. Initially, we too appreciated the opposing possibility that elevated GCs might limit inflammation and hence, reduce the development of pain-like behavior after injury. In favor of our hypothesis that prior stress would exacerbate pain, there is accumulating and compelling evidence, as described throughout this document, that stress and GCs can promote neuron plasticity and measures of inflammation. If GR is not an ideal target clinically, it is important to delineate what occurs downstream of GR activation during stress.

To this end, we have identified at least two potential cellular and molecular substrates of stress (see Fig. 22 for summary). First, we met with limited success when probing
microglia as the effector cell. Based on previous stress studies and the significance of microglia and common pro-inflammatory cytokines in neuropathic pain, we expected stress to prime neuroinflammatory processes. As such, we analyzed numerous measures of cellular reactivity, but the results were generally underwhelming. Shifting focus, we next considered the neural response to GCs. In discrete brain regions, neurons demonstrate robust susceptibility to stress and GC effects, some of which have been shown to be NMDAR-dependent. Given the role of NMDAR activation in LTP-like central sensitization in experimental neuropathic pain (Ji et al., 2003), we reasoned that stress may enhance injury-induced NMDAR activity. Indeed, an open channel NMDAR inhibitor administered prior to the stressor prevented stress effects on pain-like behavior. This, coupled with increased neuronal p-ERK, implicated neurons as superior targets for stress effects on pain.

Figure 22. Overview of Mediators in Stress-Induced Pain Enhancement. From the proposed model in Figure 1, we have confirmed that 1) stress enhances neuropathic pain-like behavior. 2nd, we have shown that prior stress utilizes both mechanisms of neuron plasticity and immune mechanisms, with the specific effectors shown in large bullets. 3rd, we have demonstrated the involvement of a few important molecular targets of stress-induced pain enhancement, including GR, MIF and mTOR.
Much of the sustained neuron plasticity that occurs after nervous system injury is unique to neuropathic pain (versus other pain types). Maybe stress exploits this plasticity to impact pain. Throughout this document, neuron plasticity is presented as measurements of function (e.g., LTP, LTD), biochemistry (e.g., p-ERK, mTOR), or structure (e.g., axon sprouting, synapse formation). In all likelihood, the neuronal microenvironment directs intracellular signaling (e.g., biochemistry) that supports diverse functional and structural dynamics. Functional neuron activity and structural plasticity are contributors to neuropathic pain. Our goal was to ascertain whether stress impacts such plasticity. In this endeavor, we learned that, indeed, acute stress increases sensory axon sprouting and elongation (Chapter 3). *In vivo*, the same effect was observed; acute stress prior to sciatic crush injury increased axon regeneration. A stress-inducible component of protein translation machinery, mTOR, emerged as the molecular target of stress in these studies (Chapter 3). Unlike GR, mTOR may represent a reasonable therapeutic target. mTOR has attracted the attention of cancer research, among others, and methods of inhibition will undoubtedly expand as a result. Unfortunately, one mTOR inhibitor, rapamycin, failed to prevent stress-induced pain enhancement in our study. Whereas the GR, MIF and NMDAR inhibitor were efficacious when administered only one time, prior to stress, the mTOR inhibitor was without effect in this capacity. So, at present, our data demonstrate clear stress-induced mTOR effects on neuron plasticity but have yet to prove its involvement in stress-induced pain enhancement.
One molecular target that kept our attention despite limited microglial data was MIF. In theory, MIF was the quintessential putative stress mediator in pain. It is highly pro-inflammatory yet induced by stress and GCs. More intriguing perhaps is its ability to counteract GCs’ anti-inflammatory mechanisms. Finally, MIF exhibits curious hormone-like characteristics for a pro-inflammatory cytokine. It is constitutively expressed in virtually every cell, circulates at comparatively high concentrations, and exhibits a circadian rhythm. Based on all of this, we hypothesized that MIF underlies stress-induced pain enhancement. In pursuing these experiments, we serendipitously discovered that MIF is essential for persistent pain, regardless of stress. MIF KO mice did not develop allodynia after peripheral nerve injury or CFA-induced hind paw inflammation. Similarly, a small molecule inhibitor of MIF reduced allodynia after nerve injury. These data provide the first evidence, to our knowledge, that MIF is important in persistent pain states. Additional experiments demonstrated a role for MIF in stress-induced pain enhancement, as expected. Together, these observations may have a strong impact on pain medicine, and more generally, on conditions such as trauma that elicit GCs.
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