Role of the sympathetic nervous system in chronic post ischemia pain, a rodent model of complex regional pain syndrome type 1

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Abstract

Complex regional pain syndrome (CRPS) is a severe neuropathic-like chronic pain disorder that is characterized by spontaneous and stimulus-evoked pain, vascular abnormalities, sudomotor changes, and muscle and bone abnormalities. Growing evidence suggests that ischemic processes may be particularly important pain mechanisms in CRPS, particularly for CRPS-type I, for which a major nerve injury is not indicated. Chronic ischemia in limbs of CRPS patients may underlie the analgesic effectiveness of anti-sympathetic treatments and vascular abnormalities.

Since most animal models of neuropathic pain involve injury of a major nerve, development and characterization of an animal model that mimicks signs and symptoms of CRPS by an ischemic injury would increase our understanding of the mechanisms underlying CRPS-I. After a 3-hour tourniquet-induced ischemia and reperfusion of the rat hind paw (chronic post-ischemia pain; CPIP), a majority of animals develop chronic pathology that mimicks CRPS-I, with long-lasting sensory symptoms of mechanical allodynia, mechanical hyperalgesia, and cold allodynia following short-lasting signs of hyperemia and oedema. Chemical sympathectomy and phentolamine reduce signs of mechanical allodynia, suggesting this animal model exhibits what has been clinically defined as sympathetically-maintained pain (SMP). Further, the systemic administration of an α_1-adrenergic antagonist, an α_2-adrenergic agonist, a nitric oxide (NO) donor, but not an α_2-adrenergic antagonist, also reduce mechanical allodynia, suggesting that vasodilation may be a way to relieve allodynia in this animal model.

The relationship between mechanical allodynia and painful reactions to intracutaneous norepinephrine (NE) injection was also measured behaviourally, and was compared with changes in NE-induced reductions in hind paw blood flow in CPIP rats. As in CRPS patients with SMP, intradermal injection of NE into the affected hind paw induced dose-dependent nociceptive behaviours. Adrenergic antagonists and a NO donor inhibited these behaviours. Intradermal hind paw injection of non-adrenergic vasoconstrictors, or NO synthase (NOS) inhibitors, also induced nociceptive behaviours in CPIP rats. Further, CPIP rats

with mechanical allodynia displayed greater NE-induced vasoconstriction in the ipsilateral hind paw as compared to the contralateral paw, as well as to sham rats, and to CPIP rats that did not develop mechanical allodynia. These results suggest parallels between mechanical allodynia and painful behavioural and enhanced vasoconstrictive responses to NE in CPIP rats. Consistent with the literature on the consequences of hind limb ischemia-reperfusion (I-R) injury and those of CRPS, CPIP pain may depend on chronic tissue ischemia that is dependent on, or exacerbated by enhanced α-adrenoceptor-mediated vasoconstriction.

Résumé

Le syndrome douloureux régional complexe (SDRC) est une douleur chronique sévère semblable à la douleur neuropathique, et se caractérise par la présence de douleur spontanée et évoquée par stimulation, des anomalies vasculaires, des changements sudomoteurs et des anomalies ossseuses et musculaires. Les recherches récentes suggèrent que les processus d'ischémie sont impliqués dans les mécanismes de la douleur du syndrome SDRC et particulièrement pour le syndrome SDRC de type I, dans lequel il n´y a pas de lésion de nerf majeure identifiable. On pense que l'ischémie chronique dans les membres des patients SDRC est reliée à l'efficacité des traitements anti-sympathetiques analgésique et aux anormalités vasculaires présentes.

Puisque la majorité des modèles animaux de la douleur neuropathique se caractérisent par une lésion de nerf majeur, on suggère que pour mieux comprendre les mécanismes pathologiques du SDRC-type I, il est necessaire de développer et de caractériser un nouveau modèle animal de la douleur chronique induie par une bléssure ischémique et qui reproduit les signes et les symptomes du SDRC de type-I. Un épisode d´ischémie suivi de réperfusion de la patte du rat par tourniquet pendant 3 heures cause chez une majorité d´animaux une pathologie chronique qui resemble au syndrome SDRC-type I (douleur chronique post-ischémique; DCPI), avec des symptomes sensoriels de longue durée comme l'allodynie tactile, l'hyperalgésie tactile, l'allodynie au froid, et aussi de l'hyperhémie et de l'oedème de courte durée. La sympathéctomie chimique et la phéntolamine diminuent les signes d'allodynie, ce qui demontre de la douleur en composant sympathique (SMP) dans ce modèle animal. En plus, l'administration systémique d'un antagoniste α_1-adrénergique, d'un agoniste α_2-adrénergique, d'un doneur de l'oxyde nitrique, mais pas d'un antagoniste α_2-adrenergique diminue l'allodynie tactile, ce qui suggère que la vasodilatation est une manière de diminuer l'allodynie dans ce modèle animal.

La relation entre l'allodynie et l´effet de l'injéction intracutanée de norépinéphrine sur les réactions douloureuses fut évalué par mesures comportementales comparé aux changements de flux sanguin en réponse à

l'injection de la norépinéphrine chez le rat DCPI. Commes chez les patients SDRC-type I avec SMP, les injections intradermalles de norépinéphrine produisent des comportements nociceptifs reliés à la dose employée. Des antagonistes adrénergiques et un donneur d'oxyde nitrique diminuisent ces comportements. L'injéction intradermalle d'une substance vasoconstrictive non-adrénergiques ou des inhibiteurs de la synthase de l'oxyde nitrique dans la patte des rats DCPI a aussi causé des comportements nocicéptifs. Chez les rats DCPI démontrant une allodynie, de plus grandes vasoconstrictions à la norépinéphrine furent observées dans la patte ipsilatérale en comparaison de la patte contralatérale, de rats sham, ou de rats DCPI n'ayant pas développé de l'allodynie. Ces résultats suggèrent qu'il existe des parallèles entre le degré d'allodynie, les comportements douloureux, et l´augmentation de la vasoconstriction en réponse à l'injection de la norépinéphrine chez les rats DCPI. Nos résultats sont compatibles avec d'autres études sur l'ischémie et la réperfusion des membres et indiquent que la douleur DCPI pourrait dépendre de l'ischémie chronique des tissus, qui résulte d'une vasoconstriction accrue dûe aux recepteurs adrenergiques-α.

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Contribution of Authors

This thesis is written in manuscript format as permitted by the McGill Faculty of Graduate and Post-doctoral Studies and is composed of three peer-reviewed published manuscripts. The contribution of each author is as follows:

Chapter 2: Coderre TJ, Xanthos DN, Francis L, Bennett GJ. Chronic post-ischemia pain (CPIP): a novel animal model of complex regional pain syndrome-type I (CRPS-I; reflex sympathetic dystrophy) produced by prolonged hind paw ischemia and reperfusion in the rat. Pain 2004; 112: 94-105.

I performed most of the experiments in this manuscript. The techniques included assessment of various methods to induce I-R injury, nociceptive testing (von Frey testing, noxious heat testing, cold testing, and pinprick testing), drug administrations, skin temperature measurements, measures of hind paw Evans Blue dye extravasation, nerve dissection and light microscopy, and statistical analyses.
Gary Bennett assisted with nerve dissection and light microscopy.
Laura Francis assisted with nerve dissection and behavioural testing.
Terence Coderre assisted with skin temperature and Evans Blue dye assessments.
The manuscript was written and prepared by Terence Coderre and edited by Dimitris Xanthos and Gary Bennett.

Chapter 3: Xanthos DN & Coderre TJ. Sympathetic vasoconstrictor antagonism and vasodilatation relieve mechanical allodynia in rats with chronic post-ischemia pain (CPIP). J Pain 2008; 9: 423-433.

I performed all of the experiments in this manuscript. The techniques included nociceptive testing (von Frey testing), drug administrations, and statistical analyses.
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Chapter 4: Xanthos DN, Bennett GJ, Coderre TJ. Norepinephrine-induced nociception and vasoconstrictor hypersensitivity in rats with chronic post-ischemia pain. Pain 2008; 137: 640-651.

I performed all of the experiments in this manuscript. The techniques included nociceptive testing (von Frey testing, noceptive behaviour scoring), drug administration, vascular catheter placements, measurement of hind paw blood flow with laser Doppler monitor probes, laser Doppler signal and trace analyses, and statistical analyses.

Gary Bennett provided valuable suggestions on optimizing experimental design, methodology, and manuscript preparation.

The manuscript was written and prepared by Dimitris Xanthos and edited by Terence Coderre.

Claims of Originality

The following original results are presented in this thesis:

Chapter 2

The goal of this study was to develop a useful animal model of CRPS-I by causing an I-R injury. Most basic pain research on neuropathic pain is done using animal models by inducing injury of a major nerve, which model CRPS-II, and there are only a handful of animal models which mimic CRPS-I. At the time of publication, none of these were well characterized. The method of tourniquet ischemia, with the use of an O-ring to produce chronic pain symptomatology, remains to this day the only animal model that examines the chronic sensory consequences of I-R injury to the hind paw, which we have called chronic post-ischemia pain (CPIP).

Chapter 3

While there have been many studies that have used anti-sympathetic treatments in animal models of CRPS-II, this is the first study that examines anti-sympathetic treatments in an animal model of CRPS-I. Further, while other studies using different animal models of CRPS-I have primarily focused on anti-inflammatory mechanisms and treatments, this is the first study to show that agents that produce vasodilatation relieve pain symptoms in an animal model of CRPS-I.

Chapter 4

Although a small number of studies have found alterations in hind paw vascular function and enhanced nociceptive responses to locally administered NE in animal models of CRPS-II, this is the first study to examine these phenomena in an animal model of CRPS-I. This is also the first study to show that enhanced NE-induced nociceptive behaviours are mimicked by non-adrenergic vasoconstrictors, paralleled by enhanced NE-induced vasoconstriction, and relieved by co-administration of vasodilators.

List of Abbreviations

ACH: acetylcholine

ANOVA: analysis of variance

ATP: adenosine triphosphate

AVP: vasopressin

cAMP: cyclic adenyl monophosphate

CCI: chronic constriction injury

CGRP: calcitonin gene related peptide

CIHR: Canadian Institutes for Health Research

CNS: central nervous system

CPIP: chronic post ischemia pain

CRPS: complex regional pain syndrome

DRG: dorsal root ganglion

EC_{50}: half-maximal effective concentration

eNOS: endothelial nitric oxide synthase

fMRI: functional magnetic resonance imaging

FRSQ: Fonds de la Recherche en Santé du Québec

GPCR: G protein-coupled receptor

i.p.: intraperitoneal

i.pl.: intraplantar

i.v.: intravenous

IASP: International Association for the Study of Pain

IL-6: interleukin 6

iNOS: inducible nitric oxide synthase

I-R: ischemia-reperfusion

L5-6: lumbar sections 5-6

L-NAME: N-(ω)-nitro-l-arginine methyl ester

L-NIO: (N5-(1-iminoethyl)-l-ornithine dihydrochloride

LSD: least significant difference

mRNA: messenger ribonucleic acid

NAC: n-acetyl-l-cysteine

NADH: β-nicotinamide adenine dinucleotide

NADPH: nicotinamide adenosine dinucleotide phosphate

NE: norepinephrine

NGF: nerve growth factor

NO: nitric oxide

NOS: nitric oxide synthase

NSAID: non-steroidal anti-inflammatory drugs

NSERC: National Sciences and Engineering Research Council

6-OHDA: 6-hydroxydopamine

PSNL or PSNT: partial sciatic nerve ligation (or transection)

PSNS: parasympathetic nervous system

PWT: paw withdrawal threshold

RSD: reflex sympathetic dystrophy

s.c.: subcutaneous

SEM: standard error of the mean

SIN-1: 3-morpholinosydnonimine

SIP: sympathetically-independent pain

SMP: sympathetically-maintained pain

SNAC: S-nitroso-acetyl-cysteine

SNI: spared nerve injury

SNL: spinal nerve ligation

SNP: sodium nitroprusside

SNS: sympathetic nervous system

SNT: sciatic nerve transection

SPGN: sympathetic postganglionic neurons

TEMPOL: 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl

TMS: transcranial magnetic stimulation

TNF-α: tumor necrosis factor-α

VEH: vehicle

XO: xanthine oxidase

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Chapter 1

1.1 Introduction

1.1.1 Chronic pain and complex regional pain syndrome

Chronic pain is one of the most common reasons for seeking health services (Von Korff et al., 1991). When people are asked to self-report lifetime incidence of any duration of pain that induced significant disability, medication use, or professional consultation, an 82% prevalence rate is reported (James et al., 1991). Clinical epidemiological studies of chronic pain in the general population estimate prevalence in the range between 2 and 40% with a median of 15% (Verhaak et al., 1998). Persistent pain is a common problem throughout the world, and often co-occurs with disability and other conditions such as anxiety and depression (Gureje et al., 1998; Gureje et al., 2001). In the recent years, basic research has achieved tremendous progress in increasing our understanding of the mechanisms in normal and pathological pain conditions. In order to optimally understand clinical chronic pain conditions, it is important to conduct research that links together both the clinical presentation with the underlying mechanisms, what has been called translational research.

CRPS, formerly known as reflex sympathetic dystrophy (RSD) or causalgia, is a severe chronic pain syndrome that often fails treatment, and is hence likely to benefit from "translational" research. Patients with CRPS typically have limbs with severe constant burning pain, mechanical and cold allodynia, swelling, skin temperature and color changes, excessive sweating, limited active range of motion, and exacerbation of symptoms with exercise. Other symptoms that occur include motor dysfunction (tremor, weakness, dystonia), dystrophy, hair and nail changes, bone changes, insomnia, and psychological/psychiatric symptom comorbidity (anxiety, depression, agitation, memory impairment). CRPS is often subdivided into CRPS-I and CRPS-II. CRPS-I is often caused by soft tissue injuries, fractures, crush, or surgery, and sometimes also spinal cord disorders, infections, stroke, heart attack, venipuncture or unknown causes. CRPS-II involves an identified injury of a major nerve and shows the same

symptoms as CRPS-I. (PARC, 2002; RSDSA, 2008; Merskey & Bogduk, 1994). The diagnosis of CRPS is sometimes controversial due to a lack of well-accepted criteria or assessments for CRPS and lack of understanding of basic mechanisms that contribute CRPS pathology, particularly for CRPS-I.

CRPS patients usually present for treatment in specialized pain clinics with severe pain and pathology that has undergone many assessments and often years of failed treatments. Only recently have systematic population studies described these patients. A survey of CRPS patients showed that these patients had seen on average 4.8 different physicians, received over 5 different treatments, and had a mean duration of pain symptoms of 30 months prior to visiting a pain clinic (Allen et al., 1999). In another study, the mean age of diagnosis was found to be 52.7 years, with a peak incidence between 60 and 70 years old, and with fracture having been the most common precipitating injury, accounting for 44% of cases (de Mos et al., 2007). Incidence statistics for CRPS have been reported between 5.46/100,000 (Sandroni et al., 2003) and 26.2/100,000 (de Mos et al., 2007). A recurring theme complicating generalizations to the general population is the small number of studies, and lack of generally accepted, and uniformly used, diagnostic criteria for CRPS (Bennett & Harden, 2003; de Mos et al., 2007; Bruehl & Chung, 2007). Research on basic mechanisms underlying CRPS will help to better understand, characterize, and raise awareness for this poorly understood disease.

1.1.2 History and overview of CRPS - causalgia and RSD

Injuries described to result in peripheral nerve damage have been reported in medical literature for at least the last few hundred years. Some of the prominently reported cases of nerve injury leading to severe pain symptomatology are popularly attributed to 16^{th}-19^{th} century European wars, initially with lance injuries and increasingly when bullet injuries became common (Bonica, 1953). Records indicate that in the 18^{th} century, the famous British surgeon Potts described how trauma can be the source of burning pain and atrophy in the extremity (Hooshmand, 1993). A surgeon named Denmark was reported to have performed one of the first amputations in 1813 for what was termed at the time as

"tic douloureux", a severe burning pain with inflammatory symptoms in a sailor as a result of a musket bullet injury (Ley, 1835). During the American Civil War in the 1860's, the famous neurologist Silas Weir Mitchell and George Morehouse, along with the surgeon W.W. Keen, examined many soldiers injured by bullets that induced nerve injury, and which suffered from a severe chronic burning pain syndrome. The soldiers also exhibited a multitude of other symptoms, such as abnormal skin color and temperature, abnormal sweating, hair and fingernail abnormalities, osteoporosis, muscle weakness and involuntary movements, as well as perceptual disturbances. With the publication of several books and articles on the subject, a disease termed causalgia, today known as CRPS-II, was first well described (LeRiche, 1939, White & Sweet, 1968). Some years later in 1900, the German surgeon Paul Sudeck noticed bone atrophy after analyzing some of the first X-rays of several painful focal limb disorder patients with a post-traumatic neuralgic syndrome. He theorized that an inflammatory pathophysiology was the mechanism, and the syndrome was named Sudeck's atrophy (Sudeck, 1900), today known as CRPS-I. Symptoms of the classical causalgia described by Mitchell were thought to be particularly rare in civilian populations (White & Sweet, 1969). In 1916, the French vascular surgeon Leriche published the first case report of successful treatment for causalgia using surgical sympathectomy (Leriche, 1916). William Livingston, an American surgeon who worked with injured soldiers of World War II, was influential in suggesting that a vicious circle of afferent input and reflex vasoconstriction, as well as disuse of the limb, pain, and atrophy could be induced with even minor nerve lesions. Although a significant proportion of patients only obtained transient pain relief, his finding that some soldiers were treated successfully with sympathectomy led Livingston to hypothesize a critical role of the sympathetic nervous system (SNS) in causalgia (Livingston, 1948). Interestingly, at about the same time, another surgeon, Philip Foisie, who was treating war-injured patients, suggested that arterial vasospasm and ischemia contributes to causalgia, or to pain following arterial or soft tissue injury (Foisie, 1947). The role of vasospasms and ischemia, which had largely been ignored until recent years, will be discussed in a later

section. The term “reflex sympathetic dystrophy” was introduced by John Evans to explain what had previously been described as a minor causalgia, in which the syndrome developed without a major nerve injury (Evans, 1946). In 1986, the International Association for the Study on Pain (IASP) included sympathetic hyperactivity as a criterion to differentiate RSD from causalgia, although it remained unclear whether that disease was simply a minor form of causalgia. However, as described below, evidence for the role of the SNS in CRPS has recently been questioned, and an expert panel consensus report advised IASP to change the names of RSD and causalgia to complex regional pain syndrome type I (CRPS-I) and type II (CRPS-II), respectively (Stanton-Hicks et al., 1995). It has been pointed out that there are historically 9 terms in English, as well as 26 terms in German and 9 terms in French, for what what has been grouped together today as CRPS (Ascherl & Blümel, 1981; in Schott, 2007).

1.1.3 Current treatment of CRPS

As with neuropathic pain, CRPS is often resistant to many analgesic treatments. Interestingly, the most commonly used pharmacological agents for pain, such as opioids, tricyclic anti-depressants, and non-steroidal anti-inflammatory drugs (NSAIDs), have not been particularly effective in CRPS patients, or have not been systematically studied (Kingery, 1997; Rowbotham, 2006; Baron, 2006). A discussion of long-used anti-sympathetic treatments for CRPS will follow in sections below. Apart from sympathetic modulation, other treatments that have been investigated and shown promise for some patients in recent years include anticonvulsants, such as gabapentin (Mellick & Mellick, 1995; van de Vusse et al., 2004), oral corticosteroids such as prednisolone (Christensen et al., 1982; Bianchi et al., 2006), sodium channel blockers, such as intravenous (i.v.) lidocaine (Wallace et al., 2000), biphosphonate compounds, such as intranasal calcitonin or i.v. clodronate (Gobelet et al., 1992; Adami et al., 1997; Varenna et al., 2000), and free radical scavenger compounds, such as topical dimethyl sulfoxide (DMSO) or oral N-acetylcysteine (NAC) (Zuurmond et al. 1996; Perez et al., 2003). Antioxidant vitamin C administration has also recently been shown to reduce the incidence of CRPS after fracture (Zollinger et

al., 1999; Amadio et al., 2000; Zollinger et al., 2007). Topical, regional, and epidural administration of the α_2 adrenergic agonist clonidine may be effective in some patients (Davis et al., 1991; Rauck et al., 1993; Reuben & Sklar, 2002) and intrathecal administration of the GABA-B agonist baclofen has been effective for CRPS-associated dystonia (van Hilten et al., 2000) Important non-pharmacological treatments that have been effective in some patients include spinal cord stimulation (Kemler et al., 2000; Taylor, 2006) and other neurostimulation techniques, such as transcutaneous electrical nerve stimulation (TENS), transcranial magnetic stimulation (TMS), and deep brain stimulation (Nelson & Stacey, 2006; Cruccu et al., 2007). Physical therapy and psychological interventions, such as cognitive-behavioural therapies, have further been shown to be helpful in CRPS, and often part of any comprehensive treatment regimen (Oerlemans et al., 1999; Lee et al., 2002; deJong et al., 2005).

The recommendations based on the literature for a standardized treatment protocol remain mixed and vague due to the few randomized controlled trials performed in CRPS, and of the diagnostic uncertainties complicating interpretation of results. Effective treatments suggest the syndrome has mechanisms that resemble both those of neuropathic and inflammatory pain. Multiple complex mechanisms remain to be disentangled, and more basic and clinical research is needed. Nevertheless, it is reasonable to conclude that a multi-disciplinary approach, such as that used in specialized pain clinics, may be most likely to bring patient benefits, since multiple suspected mechanisms of the syndrome can be targeted from multiple perspectives (Walker & Cousins, 1997; Baron, 2006; Bruehl & Chung, 2006).

1.2 Characteristics and hypothesized mechanisms for CRPS

1.2.1 Anti-sympathetic treatments in CRPS

Until recently, sympathectomy was thought to be particularly effective for treatment of patients with causalgia (White & Sweet, 1969). Its presumed effectiveness became entrenched with the accumulating reports of successful sympathectomies during the various wars in the first half of the 20th century, and this was instrumental to the initial characterization of the disease (Hooshmand,

1993). However, it is not clear if the dramatic descriptions of wartime causalgia are the same syndromes that are seen in civilian populations with nerve injuries. Nonetheless, in the 1960s, sympathectomy continued to be recommended and retrospectively described as highly successful treatment for hundreds of cases of causalgia that occurred in World War II (Richards, 1967). However, by the mid 1990's, a growing number of clinicians and scientists expressed concerns that anti-sympathetic treatments were ineffective for causalgia and RSD (Rocco et al., 1989; Blanchard et al., 1990; Jadad et al., 1995; Ochoa & Verdugo, 1995).

The existence of several different types of anti-sympathetic treatments and variability in technical success has contributed to controversy over their effectiveness. A surgical sympathectomy involves removal or destruction of the sympathetic ganglion. Destruction of ganglia can be performed via incision, radiofrequency, laser, or chemical means (traditionally with injection of alcohols or phenols). Although they have evolved since their initial use early in the 20th century, surgical sympathectomy techniques for pain are more often used as a last resort today. A temporary sympathetic blockade can also be performed by injections of a local anaesthetic, such as lidocaine, around the paravertebral ganglia that project to the affected body part. Anti-sympathetic treatments can also include systemic administration of α-adrenergic receptor antagonists, such as phentolamine, phenoxybenzamine, prazosin, or terazosin, or the regional administration of a sympathetic blocking agent such as guanethidine (sometimes bretylium or reserpine). In the seventies, Hannington-Kiff (1974) described the i.v. regional guanethidine block technique for causalgia. Regional and ganglion blocks are not always effective clinically, and when they are, they often last only for a few hours, and only a minority of patients report lifetime pain relief after surgical sympathectomy (Baron, 2006; Sharma & Raja, 2006).

Anecdotal evidence, and some research studies, suggest that sympathetic manipulation may be particularly effective only early in the disease process. Patients that undergo a surgical sympathectomy within 12 months of the precipitating injury have better outcomes in a few studies (Aburahma et al., 1994; Schwartzmann et al., 1997; Singh et al., 2003). An inverse relationship between

time to treatment initiation and pain relief has also been reported for stellate ganglion blocks (Ackerman & Zhang, 2006). However, these results have been controversial, since small sample sizes are used. Although CRPS is often only identified at late stages (when sympathetic blocks may be less effective), there are many inherent difficulties in conducting randomized controlled trials with these treatments, and in this patient population (Cepeda et al., 2002; Sharma & Raja, 2006). Further, the use of early treatment procedures on patients who would not have developed long-term CRPS, or the exclusion of early successfully-treated patients from later randomized controlled trials, may also lead to potentially inaccurate intuitive beliefs among practitioners that early treatment is most effective.

Meta-analyses of published reports of sympathetic blockade for CRPS have shown that available data is often of low scientific quality, since it is primarily based on case reports, and have concluded that anti-sympathetic treatments are not effective (Kingery, 1997; Cepeda et al., 2002; Mailis & Furlan, 2003). Controversy is also fuelled by influential studies showing that injection of saline in the sympathetic ganglia often relieves pain, possibly by placebo or another unexplained mechanism, and that there are inherent methodological difficulties in determining that sympathetic blocks are successful (Price et al., 1996; Price et al., 1998). Further, although the SNS may modulate pain, there is no evidence that hyperactivity exists to produce pain that responds to sympathetic blockade (Max & Gilron, 1999; Elam, 2001).

Apart from sympathectomy, regional and local sympathetic blocks, several early studies had also demonstrated that α-adrenergic agents may be useful in CRPS. Oral administration of the α_1-adrenergic antagonists prazosin and terazosin was reported to be highly effective in two CRPS patients (Abrams & Lightfoot, 1981; Stevens et al., 1993). Oral phenoxybenzamine was also very effective when used for causalgia in 40 missile- and shrapnel-injured patients (Ghostine et al., 1984). Muizelaar et al. (1997) also used oral phenoxybenzamine in civilian populations and found effective pain relief in 8 out of 9 early CRPS-I patients, and 7 of 17 late-CRPS-I patients. A recent study also found that oral

phenoxybenzamine was effective in 3 out of 4 CRPS-I patients (Inchiosa & Kizelshteyn, 2008). Raja et al. (1991, 1996) showed that i.v. phentolamine temporarily relieves neuropathic pain in controlled-blind studies, and that positive responses to i.v. phentolamine correlated well with pain relief from sympathetic ganglion blockade. Responses to phentolamine block also correlate well with analgesic responses to regional guanethidine (Arnér, 1991). Further, locally administered adrenergic agents are also reported to be effective for CRPS patients. Thus, Davis et al. (1991) showed that a clonidine patch was effective in 4 CRPS patients that also responded to sympathetic blockade, but not in 2 CRPS patients that didn't respond to the block.

As mentioned above, technical difficulties can complicate anti-sympathetic procedures, and it is sometimes difficult to establish the success of a sympathetic block. Changes in skin blood flow and temperature can be used to establish sympathetic blockade (Irazuzta et al., 1992). A successful Horner's or cobalt tests have been suggested as good ways to assess the success of sympathetic blockade (Stevens et al., 1998). Others have also suggested performing assessment of vasoconstrictor and sweating responses to cold challenge (Glynn et al., 1981). While problems for judging success of sympathetic blockade can exist with any of these methods, it should be kept in mind that even with a successful sympathetic blockade, there may not be sustained pain relief (Treede et al., 1992; Baron & Meier, 1996).

1.2.2 Sympathetically-maintained pain in CRPS

Based primarily on electrophysiological experiments in rodents, and reports of successful anti-sympathetic treatments in causalgia and RSD patients, Roberts (1986) introduced the hypothesis that sympathetic-sensory coupling underlies the analgesic responses to anti-sympathetic treatments in these patients. He stated that activity in myelinated mechanoreceptor afferents is induced by sympathetic efferent actions on sensory nerves, and that this afferent input causes tonic firing in previously sensitized wide-dynamic-range neurons in the spinal cord dorsal horn. According to Roberts (1986), sympathetic-sensory coupling underlies the phenomenon he coined as sympathetically-maintained pain (SMP),

which occurs due to abnormal sympathetic-sensory interaction (or coupling), and is identified when patients receive relief from anti-sympathetic treatments. While SMP has been prominently associated with limb CRPS, relief of pain by anti-sympathetic treatments has also been seen in various patients with head and neck CRPS (Giri & Nixdorf, 2007a; Giri & Nixdorf, 2007b), fibromyalgia (Martinez-Lavin, 2004; Martinez-Lavin, 2007), burn pain (Pal et al., 1997), post-herpetic neuralgia (Hogan, 1993), Raynaud's syndrome (Glynn et al., 1981), digital and palmar occlusive disease (Koman et al., 1995), angina (Hashmonai & Kopelmann, 2003), and various cancer pains (Wilsey et al., 2001). Nevertheless, sympathectomy and sympathetic blockade treatments are no longer thought to be effective standardized treatment, or to be used as diagnostic tool for CRPS. However, some subtypes of CRPS patients, and other neuropathic pain patients, do get relief from anti-sympathetic treatment, hence the term SMP remains to this day. The concept of sympathetically-independent pain (SIP) has also been termed for those patients that do not respond to sympathetic blockade, although again this is not a diagnostic entity, but is meant to guide treatment.

SMP is meant to classify patients that will receive pain relief from anti-sympathetic treatment. However, for patients that have SMP, this is suggestive of abnormal sympathetic-sensory interactions. Temperature abnormalities, blood flow changes, vasoconstrictor abnormalities, and changes in catecholamines may also be considered indirect evidence of abnormal sympathetic-sensory interactions in some subtypes of CRPS, particularly those with SMP. In one of the first studies differentiating SMP and SIP patients, Frost et al. (1988) showed that a 1-2 ^{o}C cooling stimulus with a drop of acetone resulted in pain in 9 out of 9 SMP patients, but only 5 of 14 patients with SIP, suggesting that either cold allodynia or a local vasoconstrictor response may be related SMP. A few studies have attempted to show a direct correlation between sympathetic activation and sensory symptoms. In an old study, Walker & Nulson (1948) had shown that electrical stimulation of the sympathetic chain elicited pain with a few seconds delay in 3 of 4 causalgia patients. White & Sweet (1969) also reported confirmation of this phenomenon. More recently, Drummond et al. (2001) showed that sympathetic

activation, with stimuli such as startle or forehead cooling, induced pain in about 70% of 61 mostly CRPS-I patients. Sympathetic blockade, that was deemed successful by loss of vasoconstrictor reflexes, eliminated sympathetic arousal-increased pain in 9 of 12 patients after startle, and 6 of 12 after forehead cooling (Drummond & Finch, 2004). Baron et al. (2002) showed that 12 CRPS patients separately classified with SMP or SIP show differences in their reported pain during generalized sympathetic activation by thermosuit whole body-cooling (high cutaneous sympathetic vasoconstrictor activity) and warming (low cutaneous sympathetic vasoconstrictor activity). SIP patients reported equal pain during both cooling and warming. SMP patients, on the other hand, showed significantly higher pain during cooling than warming, as well as a greater increase in the area of dynamic and punctuate mechanical hyperalgesia after cooling. There was also a significant positive correlation between amount of pain relief after sympathetic blockade and spontaneous pain elicited with the sympathetic arousal procedures. Although they had small sample sizes, these studies provide evidence for an abnormal link between the sympathetic system and pain in CRPS, particularly in those patients with SMP.

Another series of studies in CRPS patients examined the pain responses elicited by exogenously administered α-adrenergic agonists. Wallin et al. (1976) first reported such a phenomenon in four causalgic patients that had undergone a successful sympathetic blockade for their pain. Intradermal injection of norepinephrine (NE) "rekindled" the original pain and hyperalgesia that had been relieved by sympathectomy or sympathetic block. Torebjörk et al. (1995) reported that after sympathetic blockade intradermal injection of a vasoconstrictive dose of norepineprine rekindled ongoing pain and hyperesthesia in 7 out of 25 patients with SMP. As well as rekindling pain in CRPS patients after sympathetic block, NE also produces dose-dependent painful responses in CRPS patients who had been previously identified as having SMP (i.e, after the block had worn off) (Ali et al., 2000). In that study, 9 CRPS-I and CRPS-II patients were used, and 6 that received pain relief from i.v. phentolamine also showed NE-induced pain. Birklein et al. (1997), however, found that a vasoconstrictive dose of

iontophoretic NE did not elicit spontaneous pain in CRPS patients that were not screened for SMP. Recently, Mailis-Gagnon & Bennett (2004) found that intradermal administration of the α_1-adrenergic agonist phenylephrine produced burning pain and mechanical allodynia in 4 of 8 CRPS patients with SMP. Rekindling was also shown in 6 of 9 CRPS patients who had been sympathectomised. Therefore, the above results show that a subgroup of CRPS patients (with SMP) have painful adrenergic hypersensitivity to intradermally injected α-adrenergic agonists. This is also substantiated by findings that NE, at doses that elicited pain in SMP patients, does not cause any spontaneous pain or mechanical sensitivity in normal subjects (Ali et al., 2000; Fuchs et al., 2001), while a mild pain reported by phenylephrine injection in normal subjects is significantly more severe in CRPS patients with SMP (Mailis-Gagnon & Bennett, 2004). That the same pain can be rekindled with intracutaneous NE and phenylephrine after successful anti-sympathetic pain relief, may suggest that it is the skin of SMP patients at which the SNS mediates nociception. It also suggests that endogenous NE or epinephrine may be relevant to the original pain. While again the sample sizes in some of the above studies are small, they are suggestive that adrenergic sensitivity may be related to pain in some CRPS patients with SMP.

1.2.3 Blood flow changes in CRPS

Skin temperature and blood flow changes are prominent symptoms in CRPS-I patients. These changes are often included in diagnostic criteria for CRPS. Several studies have assessed the specificity and sensitivity of these criteria in identifying CRPS-I patients. While skin temperature is a reflection of circulatory activity within the skin, measures of blood flow generally assess both the microcirculation of the skin and deeper tissues, depending on the technique used. However, in general, skin blood flow and skin temperature are thought to be correlated, and skin temperature changes in response to the environment will result in sympathetically-mediated thermoregulatory processes to adjust blood flow.

Often, CRPS-I patients show skin limb temperature asymmetries with either hot or cold CRPS limbs. Using infrared thermometry, Low et al. (1994) found that 80% of CRPS-I patients show homologous side-to-side skin temperature difference of greater that 1^{o}C. Wasner et al. (2002) showed that some CRPS-I patients had on average a 2^{o}C side-to-side skin temperature difference, although this was not useful as a diagnostic tool, since more than two-thirds of the CRPS-I patients did not display these differences. Computerized thermography techniques have found 0.6^{o}C temperature differences in about two-thirds of CRPS-I patients, however, they do not differentiate CRPS-I with other pain disorders (Bruehl et al., 1996). Although thermography is more sensitive than thermometry, the reliability is only about 50% due to subjective interpretation of results (Niehof et al., 2007). Temperature asymmetries are also often not maintained within CRPS patients over the duration of the syndrome (Sherman et al., 1994). Birklein et al. (1998a) followed CRPS patients for about two years, and found that patients had a significantly hotter affected limb on first examination (5 months median disease duration), and a significantly colder affected limb on follow-up (94 months median disease duration). Therefore, clinical findings on temperature abnormalities in patients suggest that CRPS-I may involve hot and cold phases throughout the disease duration.

It is a common finding that CRPS symptoms can change over time. The general picture often reported is a short initial "hot" phase lasting weeks to months, followed by a "mixed hot/cold" phase, and finally a chronic "cold" phase lasting years. During the "hot" phase, the limb displays increased cutaneous temperature and pain, is oedematous, sweating, and inflamed, while in the "cold" phase, it shows lowered cutaneous temperature, may be hypoesthesic, is dry, and ischemic. It has been suggested that different stages can occur for varying time spans, and possibly even in different orders, during the progression of the syndrome (Bonica, 1953; Bonica, 1990). However, some authors have suggested instead that there are subtypes of CRPS patients rather than stages (Bruehl et al., 2002), while in a minority of cases, CRPS can also exist without pain (Veldman et al., 1993; Eisenberg & Melamed, 2003; Longmire, 2006). However, often, "hot"

and “cold” CRPS are differentiated in CRPS research, and may provide hints to pathophysiological mechanisms, as will be discussed later.

Altered blood flow patterns have also been demonstrated in CRPS-I patients. Like temperature changes, both increased and decreased blood flow has been measured. Christensen & Henriksen (1983) showed, a ~2 ^{o}C higher skin temperature and a 235% increase in blood flow on the affected side, using a local ^{133}Xe wash-out technique, in a group of patients with mean disease duration of 2 months. Kozin et al. (1981) also reported increased blood flow, as measured with scintigraphic radionucleide uptake, in approximately two-thirds of their patients with mean disease duration of ~6 months. Using laser Doppler and capillaroscopy, Rosen et al. (1988) found significantly decreased blood flow as compared to control subjects in CRPS patients with a mean disease duration of ~3 years. Studies have also found significant blood flow differences between the affected limb and contralateral limb. CRPS-I patients also showed early increases and later decreases when laser Doppler imaging is used to measure blood flow over a large surface area (Ackerman et al., 2005). Kurvers et al. (1995; 1996) used laser Doppler to study blood flow in patients grouped as stage I (hot CRPS-I) with a mean disease duration of ~2 months, stage II (mixed CRPS-I) with a mean disease duration of ~8 months, and stage III (cold CRPS-I) with a mean disease duration of ~36 months. It was found that stage I patients had increased skin blood flow, while stage II and III patients had decreased flow. Nutritive skin blood flow was also decreased in stage II and III patients, when measured with capillary microscopy. However, the heterogeneity of stage classification was exemplified as some patients in stage I had seven month disease duration, and some in stage III had only two month disease duration. Hence, like skin temperature, skin blood flow can be increased or decreased in the affected limb, and this may depend on the time point studied, or on the subtype of patient; although more studies report increased blood flow and temperature in the early stages, and decreased blood flow and temperature in the later stages.

1.2.4 Alterations in sympathetic efferent activity in CRPS-I

Consistent with observations of skin temperature and blood flow abnormalities in CRPS-I, various studies have also noted alterations in SNS functioning. Measuring skin temperature changes in response to central sympathetic activation gives an index of sympathetic function. Gulevich et al. (1997) used stress thermography during immersion of the asymptomatic limb in cold water and found that 93% of CRPS-I patients had excessive warming of the limb, and the specificity to the diagnosis was 87%. The use of thermal suits to induce whole-body warming and cooling cycles was also shown to elicit skin temperature differences greater than 2^{o}C between the affected and unaffected limb in 76% of CRPS-I patients tested (Wasner et al., 2002). Niehof et al. (2006) replicated this method and found greater skin temperature differences for CRPS-I patients than controls during either warming or cooling. The above results suggest that temperature abnormalities may be related to altered sympathetic vasoconstrictor activity in CRPS-I.

Changes in blood flow after various sympathetic manipulations also suggest there is abnormal sympathetic activity in CRPS-I patients. Contralateral limb cooling-induced reductions in blood flow (assessed by laser Doppler and capillary microscopy) were significantly impaired in the affected extremity in CRPS patients as compared to control subjects (Rosen et al., 1988). Birklein et al. (1998b) found that blood flow reductions produced by mental arithmetic, but not inspiratory gasp or the cold pressor test, were impaired in CRPS-I patients, as compared to control patients. Baron & Meier (1996) also found no abnormalities in inspiratory gasp-induced sympathetic vasoconstrictor activity on the ipsilateral limb in CRPS-I patients, and CRPS-I patients showed equal reductions in blood flow after head tilt, as compared with control subjects (Christensen & Henriksen, 1983). However, when patients were put in a cold room, they showed significantly decreased blood flow on the ipsilateral side, and inspiratory gasp was unable to reduce blood flow anymore in these patients (Baron & Meier, 1996).

Some of the discrepancies in the above studies may be explained by changes in sympathetic function over time in CRPS patients. Interestingly,

Schürmann et al. (1999) found a lack of blood flow changes to both cold-water challenge and inspiratory gasp in recently diagnosed CRPS-I patients, effects that diminished when patients were followed up over 3 months (Gradl & Schürmann, 2005). Wasner et al. (2001b) observed that early CRPS-I patients have higher blood flow perfusion values in the affected side as compared to the contralateral side during thermosuit body cooling and warming cycles, while chronic CRPS-I patients had generally lower blood perfusion values during warming and cooling cycles. This is consistent with findings that sympathetic vasoconstrictor activity changes during the course of the disease (Schattshneider et al., 2006). However, Kurvers et al. (1996) found sympathetic vasoconstrictor impairment, indicated by blunted blood flow changes after a cold pressor test, in either hot, cold, or mixed CRPS-I patients. Therefore, evidence suggests that impaired sympathetic function may be present in CRPS-I patients, although the ability to demonstrate it may depend on the manipulation used. It also appears that it can vary with disease progression, depending either on the stage or subtype of CRPS identified.

Interestingly, a number of studies have documented regional and systemic changes in catecholamine levels in CRPS patients. CRPS patients that respond to sympathetic blockade are shown to have a decreased venous pool of plasma epinephrine and NE on the affected limb as compared to the unaffected limb (Harden et al., 1994). Populations of exclusively cold CRPS-I patients, as well as mixed CRPS-I subjects, also exhibit decreased levels of NE and its metabolite, 3,4-dihydroxyphenylglycol, on the affected limb compared to the contralateral limb (Drummond et al., 1991; Wasner et al., 1999; Wasner et al., 2001b). Interestingly, circulating NE and epinephrine are higher in CRPS patients that respond to sympathetic blockade as compared to controls subjects. However, there is a correlation of catecholamine levels with psychological symptoms, making the authors conclude that increased levels may be more due to affective or endocrine abnormalities (Harden et al., 2004). More consistent with a localized abnormality, Drummond et al. (1996) showed a regional upregulation of α_1-adrenergic receptors in the skin of CRPS-I patients. Further, using laser Doppler blood flow measurements, it has been shown that CRPS-I patients have an

enhanced vascular responsiveness to topically applied NE (Arnold et al. 1993; Teasell & Arnold, 2004). Therefore, it appears that changes in adrenergic sensitivity are present particularly in the affected extremity of CRPS-I patients. This also suggests that some abnormalities documented in sympathetic vasoconstrictor function after central manipulations may actually be due to vascular adrenergic hypersensitivity rather to central SNS dysfunction, or that early SNS dysfunction leads to later vascular adrenergic hypersensitivity.

A small number of investigators recorded directly from nerves of CRPS-I patients. These studies found no evidence of excessive sympathetic nerve outflow (Wallin et al., 1976; Casale & Elam, 1992). However, Jørum et al. (2007) found that nociceptors of one CRPS-I patient who also responded to sympathetic blockade were hyperresponsive to locally applied NE. These studies suggest that afferent adrenergic hypersensitivity is present in at least some CRPS-I patients. While nerve recording is not easy in human populations, this technique is more common in animal models. A role for the SNS and the relation to pain has been extensively studied in animal models, which will be discussed in a later section.

1.2.5 Central mechanisms in CRPS

While CRPS presents primarily as a regional pain disorder, a multitude of central changes have also been identified and thought to be particularly important in the course of the disease. While central sensitization and neuroplastic changes are established phenomena in both neuropathic and inflammatory pain conditions, it has been suggested that particularly for CRPS-I there must be important central mechanisms involved that influence sympathetic function. It is also known that central lesions, such as those produced after stroke or spinal cord injury, can also precipitate CRPS (Wasner et al., 2003). Some authors have particularly emphasized changes in the central regulation of somatosensory, somatomotor, and autonomic activity (Jänig & Baron, 2002). Altered sympathetic activity contributing to the pain has been suggested as being part of positive feedback loops coupled with central changes. These are thought to account for both the thermoregulatory abnormalities and effectiveness of anti-sympathetic treatments in CRPS (Jänig & Baron, 2003).

Supporting evidence for central abnormalities come from findings that some CRPS patients have widespread sensory abnormalities, often hypoesthesia or spatial neglect in half of the body, or in a much greater area than the painful region (Rommel et al., 1999; Rommel et al., 2001; Drummond & Finch, 2006). In addition, CRPS patients show a reorganization of the primary somatosensory cortex (Maihöfner et al., 2003), cortical hyperexcitability as induced by transcranial magnetic stimulation (Eisenberg et al., 2005), and altered pain circuitry activation in response to pin-prick stimulation as demonstrated by functional magnetic resonance imaging (fMRI) (Maihöfner et al., 2005a). Altered motor cortex fMRI activity also appears to correlate with the degree of motor symptoms in CRPS patients (Maihöfner et al., 2007). Mislocalization of tactile stimulation is also seen in a significant proportion of CRPS patients, and this appears to correlate with stronger allodynia and altered somatosensory cortical fMRI activations (Maihöfner et al., 2006a; Pleger et al., 2006). Further, spread of painful symptoms far from the initial site of injury, even to the contralateral side, are reported in some patients (Maleki et al., 2000). While patterns of brain activation during allodynic tactile stimulation have been mapped out, it remains to be determined if they are specific to CRPS, and not to chronic pain in general (Maihöfner et al., 2006b). The same is true for sensory abnormalities such as hypoesthesia (Mailis-Gagnon, 2006). Therefore, while central changes are certainly present in CRPS, it is unclear whether they contribute to the initiation of CRPS or sympathetic abnormalities, or reflect alterations associated with long-term pathology.

1.2.6 Neuropathy and small-fiber damage in CRPS

As mentioned above, CRPS-II involves an identified injury of a major nerve, and is therefore considered a type of neuropathic pain. In CRPS-I there is no identified injury of a major nerve. While an initiating major nerve injury in CRPS-II will clearly persist upon clinical diagnosis, and is assumed to maintain the pain, this is clearly not the case for CRPS-I. A diagnosis of CRPS-I typically follows a negative neurological exam, when patients have normal nerve conduction velocities. However, recent studies provide evidence of minor nerve

injury in CRPS-I patients. Decreased myelination and sporadic axonal degeneration was observed in the sural nerves from the amputated legs of some patients with chronic CRPS-I (Van der Laan et al., 1998b). Oaklander et al. (2006) showed a ~25% decrease of normal cutaneous skin neurite density in CRPS-I patients, which was suggested to be due to small fiber axonal damage. Albrecht et al. (2006) found changes in the cutaneous innervations of the skin, sweat glands, and vasculature in two amputated limbs of chronic CRPS-I patients. While this study rekindled decade-long debates among researchers about the possible neuropathic nature of CRPS-I (Jänig & Baron, 2006; Ochoa, 2006; Rocco & Raymond, 2006), the evidence does suggest that the distinction between CRPS-I and CRPS-II may be less than initially expected. However, since many diverse injuries can initiate CRPS-I, it is doubtful that nerve injury is a prominent mechanism at least in the early stages of the disease. Interestingly, a lot of the knowledge of neuropathic pain comes from animal models that induce a major nerve injury, mimicking CRPS-II. Hence, basic mechanisms that can lead to CRPS-I symptomatology are not well characterized. Development of novel animal models that more closely resemble CRPS-I will better help in its understanding.

1.2.7 Classical and neurogenic inflammation in CRPS

Several theories have been proposed to explain inflammatory, neuropathic, and central nervous system (CNS) phenomena that are known to occur in CRPS. These are not mutually exclusive, and an important role for all of these has been suggested. As mentioned above, in the early 20th century, Sudeck's atrophy was proposed as a disease of an inflammatory pathophysiology, and this has since been incorporated as an important feature of CRPS. Recent accumulating evidence has demonstrated an important role for inflammatory mediators in the pathogenesis of CRPS. Several investigators have proposed that CRPS involves primarily an "exaggerated inflammatory response" that encompasses signs of both classical and neurogenic inflammation (Goris, 1998; Omogui, 2007; Schott, 2007).

Classical inflammation in CRPS usually includes oedema, skin hyperthermia and erythema, and pain, that all depend on mediators released from damaged tissue, as well as resident and circulating immune cells. In a study using a large CRPS population, it was shown that at any time ~88-98% of CRPS patients show pain, ~84-97% show erythema, ~55-86% show oedema, and ~89-98% display hyperthermia (Veldman et al., 1993). In addition, CRPS patients have higher levels of interleukin-6 (IL-6), tumour necrosis factor α (TNF-α), and leukocytes in fluid removed from experimental skin blisters of the ipsilateral CRPS limb, as compared to the contralateral limb (Huygen et al., 2002; Huygen et al., 2004; Tan et al., 2005). Upregulated cytokines interleukin-1β (IL-1β) and IL-6 are also seen in the cerebrospinal fluid of patients, but are not found in blood (van de Beek et al., 2001; Alexander et al., 2005). Increased interleukin-2 and TNF-α messenger ribonucleic acid (mRNA) levels in systemic venous blood, as well as a correlation of elevated plasma TNF-α levels and mechanical hyperalgesia in plasma, has also been shown in CRPS-I patients (Maihöfner et al., 2005b; Uçeyler et al., 2007). There is a greater prevalence of inflammatory symptoms earlier in the disease, in what is usually described as the "hot" phase (Oyen et al., 1993). Some studies have further shown that late phase "cold" CRPS patients do not show upregulated cytokines, and skin blister fluid cytokine levels are not increased in the affected extremity in patients that have had CRPS for more than two years (van de Beek et al., 2001; Munnikes et al., 2005). Therefore, it has been speculated that inflammation may be part of the initiating pathophysiological mechanism of CRPS, although the relation to long-term CRPS pain remains to be determined.

Neurogenic inflammation has also been shown as an important contributing factor in CRPS. Activation of C-fibers results in secretion of neuropeptides such as calcitonin gene-related peptide (CGRP) and substance P from their peripheral terminals. These released substances cause plasma extravasation, vasodilatation, and various trophic effects in peripheral tissues. Some of the end-results may be pain, sweating, oedema and excessive hair growth, and a potentiation of classical inflammation (Birklein, 2005). Studies

have shown that serum CGRP and substance P levels are indeed increased in CRPS patients (Birklein, 2001; Schinkel et al., 2006). Exogenous intradermal substance P application induces significantly greater plasma protein extravasation in both the affected and unaffected limb of CRPS patients (Leis et al., 2003). Further, electrical stimulation of C-fibers evokes enhanced neurogenic inflammation in CRPS patients (Weber et al., 2001). The above experiments all suggest that both classical and neurogenic inflammatory processes are important in CRPS, perhaps more so in the early, hot phase.

1.2.8 Ischemia and vascular abnormalities in CRPS

Accumulating evidence has been emerging that patients with CRPS also show vascular abnormalities indicative of ischemia. While vascular abnormalities are particularly prominent on dermatological examination in some CRPS-I patients (Sundaram & Webster, 2001), ischemic processes are also evident with changes in skin temperature and with measures of blood flow (Jänig & Baron, 2003). Chronic CRPS limbs show decreased skin capillary haemoglobin oxygenation (Koban et al., 2003), thickened capillary membrane layers (Van der Laan et al., 1998b), and enhanced anaerobic glycolysis as evidenced by increased skin lactate and decreased adenosine triphosphate (ATP) levels (Heerschap et al., 1993; Birklein et al., 2000c). Experimental acidosis induces exaggerated pain in CRPS patients (Birklein et al., 2000b) suggesting ischemic low pH may be related to CRPS pain. CRPS limbs also show evidence of high peripheral venous pressure suggestive of oedema and arteriovenous shunting (Matsumura et al., 1996; Schürmann et al., 2001b), a finding seen after experimental I-R injury (Kennedy et al., 1981). A chronic and persistent regional ischemic state will very likely be painful via a variety of mechanisms. These will be discussed more in detail in the upcoming sections.

Increasing clinical research has also further characterized vascular abnormalities in CRPS. Several studies have examined endothelial function and regional vasodilatory capacity in CRPS-I patients. Using iontophoresis, Gorodkin et al. (2004) did not find significant ipsilateral to contralateral differences in acetylcholine (ACH) or sodium nitroprusside (SNP)-induced vasodilatation in

CRPS-I patients. However, when Schattschneider et al. (2006b) selected CRPS-I patients with cold limbs, they did find impaired vasodilatory responses to ACH, but not SNP, suggesting endothelial dysfunction is present in this group of patients. Measures in blister fluids of cold CRPS-I limbs also showed lowered NO levels and increased endothelin-1 in these patients (Groeneweg et al., 2006), although endothelin-1 was not altered in venous plasma of CRPS-I patients (Eisenberg et al., 2004). Duman et al. (2008) found that vasodilatation associated with reactive hyperaemia after brief tourniquet application was not altered in early stage CRPS-I patients, with an average of 5 months disease duration. However, they did find altered patterns or waveforms during vasodilatation in all CRPS patients indicative of some type of vascular abnormality. Therefore, these studies suggest some form of functional vascular abnormality, perhaps in chronic patients, although more studies are needed to clarify the mixed results.

Only a couple of studies have been able to thoroughly examine the vasculature in limbs of CRPS-I patients. Van der Laan et al. (1998b) found that skeletal muscles of the amputated legs of eight CRPS-I patients consistently showed shrunken endothelial cells and abnormal capillaries as evidenced by multiplication of the basal membrane or a severely thickened basal membrane. Albrecht et al. (2006) recently examined the skin of the amputated limbs of 2 chronic CRPS-I patients. They found clear evidence of vascular abnormalities with greatly decreased sensory peptidergic innervations to all cutaneous arteries, as well as some regional decreases in sympathetic and neuropeptide Y innervations. Dermal blood vessels displayed thickened multi-laminar walls and enlarged lumens. Endothelial cell abnormalities were also evidenced by abnormal binding with endothelial cell adhesion markers. While this study was comprehensive anatomically, the patients used were very severe chronic cases and it is not clear how much they generalize to the time course and heterogeneity of the disease. Nevertheless, the data provide support for focusing on vascular dysfunction as a source of pathophysiology in CRPS-I.

1.3 Ischemia, ischemia-reperfusion injury and pain

1.3.1 Ischemic conditions and tourniquet induced-ischemia during surgery

Ischemia is well known to result in both acute and/or chronic pain in both experimental and clinical settings. Ischemic states occur in various long-term conditions such as angina, critical limb ischemia or Raynaud's syndrome. While these are usually not classified as chronic pain disorders, pain can be a prominent symptom in all of these. Acute thrombotic, embolic events, trauma, iatrogenic injury, aneurysm, and aortic dissections can also all result in various degrees of acute, chronic, or permanent limb ischemia with accompanying persistent pain. Consequences of ischemia are particularly important during surgery. Controlled ischemic states are often induced, with the use of tourniquets or vascular occluders, to reduce blood loss and/or provide good operating conditions in limb extremity and reconstructive surgeries, transplants, and during major trauma. I-R injury, compartment syndrome, and chronic pain disorders are several of the recognized post-ischemic consequences that can occur even after brief ischemic episodes (tens of minutes to hours) (Callum & Bradbury, 2000). Further, prolonged tourniquet application or ischemic occulsion (several hours or more) can result in severe consequences, with the extremes being limb amputation or even death due to tourniquet shock and a systemic inflammatory response syndrome that leads to multiple organ dysfunction.

While a safe guideline for arterial occlusion in surgery is usually up to 2 hours of continuous complete ischemia, case reports highlight significant variability with occasional development of CRPS-I, or nerve injury and CRPS-II, after lower ischemic time periods (Bolton & Mcfarlane, 1978; Landi et al., 1995; Ferkel et al., 1996; Kornbluth et al., 2003). Retrospective analyses of arthroscopic and other surgeries reveals that patients subjected to tourniquets, usually longer than 60 minutes, are more likely to develop CRPS and various painful symptoms (Sherman et al., 1986; Berga et al., 2002). It is understood that various factors, other than ischemic duration, can also influence the extent of I-R injury, including temperature, physician training, types of tourniquet used, and the extent of the ischemic region (Kam et al., 2001; Kragh et al, 2007).

Consequences of prolonged clinical tourniquet use can involve both systemic and local effects particularly due to I-R injury. Depending on the duration of ischemia and the volume of body area occluded, systemic effects can occur during ischemia and/or reperfusion. These include cardiovascular effects (increase in heart rate and blood pressure), respiratory effects (increase in end-tidal carbon dioxide tension), cerebrovascular effects (increase in cerebral blood volume) (all during reperfusion), haematological effects (hypercoagulability during ischemia and increased thrombolytic activity during reperfusion), core body temperature changes (increase during ischemia and decrease during reperfusion), and metabolic changes (increased lactate and decreased blood pH during ischemia and reperfusion) (Kam et al., 2001). Local effects, often relating to the compression underneath the tourniquet and ischemia distal to it, can also occur depending on the tourniquet pressure and duration of ischemia. The main tissues affected underneath the tourniquet are nerve and muscle, with nerve more affected by amount of pressure, while muscle is affected more by the duration of ischemia. Skin and tendon damage are also potential complications, although these tissues are thought to be more resistant. Vascular abnormalities relating to oedema, no-reflow, and arteriovenous shunting are also documented with increasing ischemic times. Clinical recommendations generally suggest pressures between 200 and 300 mm of Hg with influencing variables including patient characteristics, limb type, and tourniquet shapes (Estebe & Mallédant, 1996; Kam et al., 2001).

1.3.2 Experimental tourniquet-induced ischemic pain in humans

Short periods of tourniquet ischemia are often used as acute pain measures in human volunteers for the study of ischemic pain. It is known that both a wider cuff and a larger ischemic limb area cause more pain (Estebe et al., 2000; Karalezli et al, 2007). In awake subjects, pressures as low as 100 mm Hg evoke progressively increasing pain within minutes of tourniquet inflation on the upper mid-arm level, which can usually only be tolerated for 20 to 30 minutes. Upon tourniquet deflation, pain relief occurs briefly for seconds, but a reperfusion burning and throbbing pain then occurs that lasts minutes (Crews et al., 1991).

The mechanisms of pain that occur during the tourniquet inflation, and after its deflation, are not necessarily the same. Pain during the tourniquet is thought to be due to local pressure on the skin since topical anaesthetics can attenuate pain (Tsai et al., 1993). In animal studies, the tourniquet is shown to directly activate sensory nerves and result in increased activity of spinal dorsal horn neurons and of rostroventral medulla cells (Crews et al., 1994; Crews & Cahall, 1999). Skeletal muscle afferent activation during an ischemic state is also thought to be painful, and is possibly due to the accumulation of metabolites such as lactate (Mense, 1993). Pain after tourniquet release is due to the reperfusion processes, with increasingly longer ischemic periods resulting in more severe symptomatology. Brief experimental tourniquet limb ischemia for minutes is unlikely to cause the vast complications of I-R injury that occurs after hours of ischemia. Nevertheless, it is interesting to note that a 10 minute forearm ischemia in human volunteers results in an increased inflammatory response and coagulation activity, as detected by *ex vivo* immunological changes in leukocytes and monocytes. Most changes persist for 5-15 minutes post-reperfusion, although some last for 24 hours. Impaired post-reperfusion vasodilatation, suggestive of endothelial dysfunction, has also been seen after a brief 30-minute ischemia (Kilian et al., 2005; Hughes et al., 2006; Hughes et al., 2007).

1.3.3 Tourniquet-induced ischemic pain in animals

Early animal models have systematically measured nociception after brief ischemia using a tourniquet around the rat tail. Awake restrained rats were subjected to ischemia of the rat tail (200 mm Hg) until they displayed escape behaviours (approximately 12 minutes). This procedure induced decreased tail flick latencies and mechanical hypersensitivity for at least 1.5 hours post-reperfusion (Gelgor et al., 1986a; Vidulich & Mitchell, 2000). Electrophysiological responses of thalamic neurones active during the ischemia correlate with the heat hyperalgesia following the reperfusion (Gelgor et al., 1988). The model was modified by Grace et al. (2001) who used a standardized 20 minute ischemia, but found only 30 minutes of thermal hyperalgesia with this protocol. Increased prostaglandin synthesis is seen in the tail and brain after tail

ischemia, but does not correlate well with hyperalgesia or with excitability of dorsal horn neurones (Grace et al., 2001; Gelgor & Mitchell, 1995). Tail ischemia-induced hyperalgesia is reduced with aspirin (Gelgor et al., 1986b), NSAIDS (Gelgor et al., 1992; Grace et al., 2001), n-methyl-D-aspartate (NMDA) antagonists, benzodiazepines, and tramadol (Sher et al., 1992; Cartmell & Mitchell, 1993; Loram et al., 2007). The large number and diversity of agents effective in this model suggests the pain is not neuropathic, and may also involve other cognitive components related to handling stress and escape behaviour. Since this model exhibits transient hyperalgesia after brief ischemia, it can be hypothesized that such short ischemia times do not involve significant I-R injury contributing to the pain symptomatology.

Seasholtz et al. (2001) studied the physiological consequences of an ischemic time of 60 minutes via arterial occlusion of the tail in anaesthetized rats. At 60 minutes post-reperfusion, they found evidence of vascular abnormalities including increased contractility of the tail artery to NE. Increased inositol phosphate production was also shown, and the increased contractility was suggested to be due to enhanced G-protein coupling in α_1-adrenergic receptors. While this study did not examine any behaviour in these animals, it does suggest that significant alterations in vascular function can occur with just 60 minutes of ischemia.

1.3.4 Animal models of prolonged hind limb ischemia and ischemia-reperfusion injury

Cellular hypoxia associated with ischemia is known to result in a diversity of changes that can ultimately result in apoptosis or necrosis in various tissues. Some of these include impaired mitochondrial function, increased free radical production, early increase and later inhibition of antioxidant enzyme activity, nuclear factor-κ-B activation, increased cellular permeability, heat shock protein induction, and multiple protein kinase activations (Li & Jackson, 2002). I-R injury has been studied particularly in heart, liver, intestine, skin flaps, skeletal muscles, and whole limbs. Although it is well known that various tissues are differentially resistant to ischemia, most of the alterations described above have been

demonstrated, and can be assumed to occur across all tissues. Very old speculations to account for I-R injury have been that toxic metabolites accumulate during ischemia and are released in the circulation with reperfusion (Perry, 1994; Khalil et al., 2006). More recently, a major influential mechanism for reperfusion was described by McCord (1985), who suggested that various oxidases, accumulate during ischemia and trigger the production of oxygen free radicals during reperfusion, triggering a cascade of events that result in I-R injury. Korthuis et al. (1989) showed that the return of oxygenated blood during muscle reperfusion results in more severe vascular injury than the return of anoxic blood. This has suggested a major role of reactive oxygen species in I-R injury, which can combine with other ischemic mediators, such as NO or hydrogen peroxide, to form additional free radical products, such as peroxynitrite or hydroxyl radicals. Enzymes such as xanthine oxidase (XO) or nicotinamide adenosine dinucleotide phosphate (NADPH) oxidase, known to increase in ischemic skeletal muscle, contribute to production of free radicals by reducing molecular oxygen (Akimitsu et al., 1994). Injury produced by free radicals causes neutrophil and leukocyte infiltration, activation of macrophages and release of inflammatory mediators such as cytokines, all of which can also lead to further free radical production (Gute et al., 1998; de Groot & Rauen, 2007). All these processes are thought to interact and be self-reinforcing long after the initial ischemia, resulting in temporary or prolonged tissue dysfunction.

Hind limb I-R injury is typically induced in rabbits or rats using thigh level tourniquets that produce ischemia for several hours with pressures ranging from 100 to 1000 mm Hg. Two hours or more of ischemia with pneumatic tourniquets at 350 mm Hg on the thighs of rabbit induced marked histopathologic muscle abnormalities beneath the tourniquet detectable at 2 days post-reperfusion (Pedowitz et al., 1990; Pedowitz et al., 1991a). Muscle injury was seen in the leg muscles of half the animals, although no regional necrosis distal to the tourniquets was found at 2 days post-reperfusion (Pedowitz et al., 1992). With the same pressure and time, muscle contractile strength distal to, and under, the tourniquet has been shown to be impaired up to 25% of control for at least 2 days post-

reperfusion (Lieber et al., 1992; Jacobson et al., 1994; Ohara et al., 1996). These functional impairments reverse slowly until 3 weeks post-reperfusion, and are worse directly underneath the tourniquet (Fish et al., 1989; Mohler et al., 1999). A 2-hour, 350 mm Hg thigh tourniquet also induces decreased nerve conduction velocity in the thigh nerve underneath the tourniquet for up to 2 days post-reperfusion, but only for 1 hour in the leg nerve distal to the tourniquet. No consistent degeneration of nerve fibers was observed with either light or electron microscopy (Pedowitz et al., 1991b). However, two hours of thigh tourniquet ischemia produced a 15% degeneration of sciatic nerve fibers when the pressure is 300 mm Hg, and 45% degeneration when the pressure is 400 mm Hg, one week post-reperfusion in rats (Pedowitz, 1991). There was less degeneration at 3 weeks post-reperfusion (10.7% and 27.3 % for the two pressures, respectively), and even less at 6 weeks (8% and 17.8%, respectively) (Nitz et al., 1986; Nitz et al., 1989). Interestingly, short ischemic times of minutes to 2 hours with pneumatic tourniquets in human volunteers produced transiently decreased nerve conduction velocities that resolved after minutes or hours after reperfusion, depending on length of ischemia (Nielsen & Kardel 1974; Rorabeck, 1980, Hurst et al., 1981). Therefore, whole limb ischemia with a pneumatic thigh tourniquet can result in muscle and nerve dysfunction, particularly underneath the tourniquet, that is reversible to various degrees, if ischemia is not prolonged.

In recent years, researchers have used thigh tourniquets which minimize neuromuscular damage due to crush injury. For example, Bonheur et al. (2004) introduced the controlled tension tourniquet, which uses a rubber band, pulleys, and a winch to control tension. Using this method, 3 hours, but not 1 hour, of hindlimb ischemia induced a significant reduction in mitochondrial activity (an index of hypoxia) in the ischemic limb tissues at 24 hours post-reperfusion. Crawford et al. (2007) used orthodontic rubber bands around the thigh of rats and found them to induce minimal tension during ischemia and less neuromuscular injury, as compared to the McGivney haemorrhoidal ligator (a metal ring), but nevertheless produced complete ischemia throughout the ischemic period as measured by laser Doppler. Orthodontic rubber bands resemble O-rings and also

show reproducible I-R injury, with 2.5 hours of hind limb ischemia inducing decreased tissue mitochondrial activity at 24 hours post-reperfusion. Systemic effects were also evident with mortality in some animals and tail perfusion changes early after reperfusion, as well at 24 hours. Sensory abnormalities, such as nociception, have not been systematically investigated long-term after this type of ischemia.

1.3.5 Vascular alterations after hind limb ischemia-reperfusion injury

One particularly important peripheral abnormality of I-R injury is long-term changes in vascular function. These include changes in vascular tone and reactivity, arterial vasospasms, and capillary no-reflow. Vascular abnormalities themselves reinforce long-term I-R injury. Endothelial cell dysfunction is a well established phenomenon after I-R injury (Conger & Weil, 1995; Carden & Granger, 2000; Seal & Gewerz, 2005). Thus, 1-2 hours of hind limb ischemia in mice and rats results in significant activation of endothelial cell adhesion proteins, changes in vascular permeability, and decreases in capillary density (Kyriakides et al., 2000; Wolfard et al., 2002). Capillary no-reflow, generally defined as zones of hypoperfusion following ischemia to a region due to damaged and plugged capillaries, has been widely studied particularly in the context of cardiology, transplants and reconstructive surgery. However, the phenomenon has also been demonstrated after prolonged limb ischemia (Blaisdell, 2002; Allen et al., 1995; Reffelmann & Kloner, 2006). Decreased flow or no-reflow in capillaries was first observed in skeletal muscle of rabbits after prolonged limb ischemia (Harman; 1948). Since then, a wide range of mechanisms have been suggested to explain the consistently observed vascular abnormalities that occur after various I-R injuries, which include: arterial spasm, oedema, endothelial and parenchymal cell swelling, increased capillary permeability with fluid extravasation and increased blood viscosity, thrombus formation, arteriovenous shunting, and leukocyte plugging (Kennedy et al., 1981; Korthuis et al., 1985; Mazzoni et al., 1995; Harris et al., 1997; Nanobashvilli et al., 2003). Interacting mechanisms such as hypoxic oxidative stress and inflammatory processes (as briefly discussed above) have also been implicated in reinforcing these changes and maintaining a chronic

ischemic state long after the initial ischemia (Allen et al., 1995; Menger et al., 1997; Gute et al., 1998; Blaisdell, 2002).

Many studies have also demonstrated chronic alterations in the vascular reactivity to vasoactive agents after I-R injury in various tissues. These alterations may be due to endothelial cell and smooth muscle cell dysfunction, which result in impaired vasodilatation and enhanced vasoconstriction. The combination of impaired vasodilatation and enhanced vasoconstriction leads to reduced blood flow associated with arterial vasospasms, and may serve to contribute to longer-term abnormalities such as capillary no-reflow, discussed above. One key mediator of vascular function is NO, due to its strong vasodilatory and anti-aggregatory properties (Moncada & Higgs, 2006). Reduced NOS activity, particularly of the neuronal and endothelial NOS subtypes (nNOS & eNOS), is thought to occur after I-R injury. Several studies show that NOS inhibitors are unable to act on their substrate after I-R injury (Laude et al., 2001; Garcia-Villalon et al., 2005). It is also known that reduced NO produced by inducible NOS (iNOS) activity is sequestered to produce nitrogen free radicals, which in turn results in further reduction of NO-induced vasodilatory activity, causing arterial vasospasms (Mehta et al., 1989; Forman et al., 1998; Zhang et al., 2003; Qi et al., 2004). Vasospasms have been shown after experimental I-R injuries in various tissue types including skeletal muscle (Seal & Gewerz, 2005), but are also reported after clinical tourniquet use (Gazmuri et al., 2002). While a normal endothelium is known to respond to substances such as ACH in order to stimulate NO release (Furchgott et al., 1987; Vanhoutte, 1988), damage to endothelium is known to induce altered vascular reactivity, such as increased vasoconstriction or reduced vasodilatation to various vasoactive agents (Ku, 1982). Hence, after limb I-R injury, alterations in vascular reactivity are consistent with endothelial dysfunction.

Many studies have found altered vascular reactivity after I-R injury and pathological changes can be reversed with NO supplementation. In the isolated rat hind limb, ACH-induced dilation is impaired after 60 minutes of ischemia and 10 minutes of reperfusion (Sternbergh et al., 1993). This is also found after a 2-hour

canine femoral artery occlusion, followed by 90 minutes of reperfusion (Joviliano et al., 2005). Three-hour complete vascular occlusion in rabbit hind limbs also produces impaired ACH relaxation of isolated femoral arteries, when at least 2 hours of reperfusion is allowed, but not earlier (Chiang et al., 1996). One hour after a 3-hour canine femoral artery ischemia, the isolated artery shows decreased half-maximal effective concentration (EC_{50}) values for NE-induced vasoconstriction, and increased EC_{50} values for SNP-induced vasodilatation (Sapienza et al., 1996). There is also arteriole vasoconstrictive hyper-reactivity to NE after 2 hours of ischemia and 1 hour of reperfusion in the rat cremaster muscle (Lee et al., 1995). Infusion of the NO donor SNP, but not ACH, can reverse a vasospasm observed after 4-5 hours of ischemia and reperfusion of the rat cremaster muscle. NO supplementation can also reverse an artificial NE-induced vasospasm in the same preparation (Wang et al., 1995; Pemberton et al., 1996; Wang et al., 1997). It is also reported that L-arginine, the NO precursor, can restore NO levels that are decreased post-reperfusion, and can improve blood flow, in rabbits subjected to 2.5 or 6 hours of hind limb I-R injury (Huk et al., 1997; Blebea et al., 1996; Nanobashvili et al., 2004). Administration of the NO precursor L-arginine a few minutes before reperfusion (Meldrum et al., 1999) reduces the necrosis that occurs 24 hours after 4 hours of ischemia and reperfusion in the rodent skeletal thigh muscle. Systemic administration of the NO donor S-nitroso-acetyl-cysteine (SNAC) also improves contractility of 3 hour ischemic rat extensor muscle (Chen et al., 1998), and local application or intra-arterial administration of SNAC improves muscle contractility and improves blood flow by inhibiting vasospasm after 4-5 hour cremaster muscle ischemia (Wang et al., 1997; Liu et al., 1998). Intra-arterial administration of the NO donor 3-morpholinosydnonimine (SIN-1) increases viability and survival of pig skin flaps subjected to 6 hours of ischemia (Khiabani & Kerrigan, 2002), and reverses abnormal endothelial-dependent ACH relaxation after 3-hour rabbit hindlimb ischemia (Johnson et al., 1998). Finally, intra-arterial L-arginine improves impaired ACH endothelium dependent vasodilatation that occurs after a brief 20 minute tourniquet I-R injury in human volunteers (Pernow et al., 2003). These

results suggest that impairment of NO vasodilatory function is an important contributor to endothelial cell and vascular dysfunction produced by I-R injury.

It is interesting to note that animals with artificially-induced chronic (weeks) limb ischemia used to model human chronic ischemic diseases, also display evidence of altered vascular reactivity. For example, arterioles from rabbits and dogs with chronic limb ischemia show blunted ACH-induced vasodilatation and enhanced serotonin-induced vasoconstriction (Orlandi et al., 1986; Bauters et al., 1995). Arterioles from rats with chronic limb ischemia also show blunted ACH-induced vasodilatation starting 2 weeks post ischemia, and reduced SNP-induced vasodilatation starting at 5 weeks post-ischemia (Kelsall et al., 2001). Skeletal muscle resistance arteries also show hypersensitivity to NE on the ischemic side, as compared to contralateral side, in rats with chronic limb ischemia (Bund et al., 1991). Clinically, critical limb ischemia patients show abnormalities in vascular function. For example, there is decreased forearm vasodilatation or reactive hyperaemia in response to removal of a briefly applied tourniquet (Yataco et al., 1999), and increases in NOS inhibitor metabolites, suggestive of decreased NO formation (Boger et al., 1997). There is also hyper-reactive vasoconstriction to NE in the proximal arteries of skeletal muscle in patients with critical limb ischemia, while the more distal arteries show hyporeactivity (Hillier et al. 1999; Coats, 2003). Another study in such patients found no such differentiation, and showed that skeletal muscle arterioles are hypersensitive not only to NE, but also to α_1- and α_2-adrenergic agonists (Jarajapu et al., 2001a). These findings complement acute ischemia studies, and support findings that I-R injury results in changes in vascular reactivity. These can be demonstrated as both hypersensitivity to vasoconstrictors or impaired vasodilatation, mechanisms that would both serve to maintain chronic pathology.

1.3.6 The role of nitric oxide in neuropathic pain and CRPS

In general, animal models have suggested that NO plays a pro-nociceptive role, but this is controversial. Intrathecal SIN-1 has been shown to evoke mechanical hyperalgesia acutely (Przlewocka et al., 1994), and systemically administered nitroglycerin has been reported to decrease tail-flick latencies

starting at two to four hours after administration when the dosage is in the milligram range (Tassorelli et al., 2003), but not in the microgram range (Masue et al., 1999). The pro-nociceptive effects of systemic administration of nitroglycerin is likely due to central and brain mechanisms, since peripheral distribution of nitroglycerin is thought to occur immediately (Torfgard & Ahnler, 1991). In rat nerve injury models, it has generally been found that NO is pro-nociceptive, but this is controversial. Intraperitoneal (i.p.) administration of the NO donors SNP and S-nitroso-N-acetylpenicillamine (SNAP), or the NO precursor L-arginine, in chronic constriction injury (CCI) rats increase mechanical and heat hyperalgesia between three and seven hours after their administration (Naik et al., 2006b), although results of early time points were not reported. Low doses of i.t. SIN-1 have been reported to reduce mechanical allodynia after CCI, while higher doses had no effect, and even higher doses aggravated mechanical allodynia which was reported to occur always with a delay as compared to the anti-nociceptive effects of low doses (Sousa & Prado, 2001).

Most studies, however, have observed anti-nociception after reducing NO using NOS inhibitors. Intrathecal N-(ω)-nitro-l-arginine methyl ester (L-NAME), a non-specific NOS inhibitor, reduces thermal hyperalgesia in CCI rats (Meller et al., 1992). L-NAME (i.p or i.t.) or a neuronal NOS inhibitor 7-nitroindazole (7-NI) also reduces both cold and mechanical allodynia in rats with spinal nerve ligation (SNL) (Yoon et al., 1998) and mice (Guan et al., 2007), but also see Luo et al. (1999). However, there was no significant reduction in mechanical allodynia in SNL rats after i.t. administration of another non-selective NOS inhibitor L-NG-monomethyl-L-arginine (L-NMMA), or a selective neuronal NOS inhibitor TRIM (Pan et al., 1998). Lee et al. (2005) further suggested that the anti-nociceptive effects of L-NAME may not be mediated by NO in SNL rats, because L-arginine cannot reverse them. Another series of studies suggested that anti-nociceptive effects of early L-NAME administration in CCI rats may be by reducing upregulation of endothelial NOS (eNOS) which upregulates early in injured axons. The early NO generation is thought to correlate with pain and initial

hyperaemic response after injury (Levy & Zochodne, 1998; Levy et al., 2000; Levy & Zochodne, 2004).

Interestingly, in human studies, intracutaneous and paravascular injections of NO solutions evoke pain in human volunteers, but infusion of local NO donors do not (Holthusen & Arndt, 1994; Holthusen & Arndt, 1995). However, i.v. infusion of the NO donor nitroglycerin is well-known to specifically evoke spontaneous headaches and migraines in humans (Olesen et al., 1994). On the other hand, NO donors have been widely utilized for angina pain, but also used as anti-nociceptive agents in various other human experimental trials for acute and chronic pains, including CRPS. Some studies report headache and migraine as a limiting factor for clinical use. In human skin, thermal hyperalgesia induced by capsaicin is aggravated by NE vasoconstriction, and this effect is reversed by vasodilatation with the NO donor SNP (Drummond, 1999). Topical nitroglycerin is well known to reduce pain associated with angina and peripheral vascular disease (Fletcher et al., 1997), and SIN-1 has also shown effectiveness for ischemic pain when given orally (Messin et al., 2006). Topical glyceryl trinitrate also promotes vasodilatation and relieves pain associated with Raynaud's syndrome (Teh et al., 1995). Topical NO donors also relieve pain associated with tendinopathy of the elbow (Paoloni et al., 2003), tendinopathy of the Achilles heel (Paoloni et al., 2004), and in anal fissure (Lund & Schonefield, 1997). Patients with painful diabetic neuropathy have also been treated successfully with NO donor administration in spray or patch form in several randomized controlled trials (Yuen et al., 2002; Rayman et al., 2003; Agrawal et al., 2007). In CRPS-I, there have been case studies that show transdermal nitroglycerin is effective for pain (Hyland, 1989; Manahan et al., 1993). Recently, a preliminary trial in patients with cold-type CRPS-I showed improvement in 3 of 5 patients (Groeneweg et al., 2008).

1.4 Animal models of CRPS

1.4.1 Animal models of CRPS-II

Various animal models of neuropathic pain have been developed that exhibit similarities to the human condition of CRPS-II. Neuropathic animal models include diabetic-induced neuropathy (Courteix et al., 1993), inflammatory (Eliav et al., 1999), and chemotherapeutic (Tanner et al., 1998) neuropathy, but it is those that use traumatic nerve injury that are most relevant for CRPS-II. Most prominently used traumatic nerve injury models include sciatic nerve transection (SNT) (Wall & Gutnik, 1974), partial sciatic nerve transection/ligation (PSNT or PSNL; Seltzer et al., 1990), chronic constriction injury (CCI; Bennett & Xie, 1988), spinal nerve ligation (SNL; Kim & Chung, 1992), and spared nerve injury (SNI; Decosterd & Woolf, 2000). Although traumatic nerve injuries were developed to model neuropathic pain, they all exhibit characteristics that suggest they also model CRPS-II.

Several studies using animal models of neuropathic pain have observed important vascular abnormalities, as well as changes in hind paw temperature and blood flow, which would be particularly relevant to CRPS-II. About 31% of CCI rats show 0.8^{o}C higher temperatures in the ipsilateral limb as compared to the contralateral limb temperature early after CCI injury, while 11% of rats show the opposite pattern when tested at 8 to 10 days post-surgery (Bennett & Ochoa, 1991). Others support this finding, showing that the ipsilateral limb is hotter than the contralateral limb for the first few days, while several days to weeks thereafter, the ipsilateral limb is significantly colder than the contralateral limb (Wakisaka et al., 1991; Hord et al., 1999). As measured with laser Doppler skin probes, there is also an increase in limb blood flow in the first few days after CCI surgery, and decreases in blood flow for several weeks after nerve injury (Gonzalez-Darder & Segura-Pastor, 1994; Kurvers et al., 1997; Hord et al., 1999). Impairment of the sympathetic vasoconstrictor response induced by abdominal cooling is also seen on the ipsilateral limb of CCI rats (Kurvers et al., 1997). The vasodilatory responses to local application of the NO donor, SNP, are significantly decreased in the ipsilateral paw of CCI rats (Basile et al., 1993).

Three weeks after CCI, isolated subcutaneous (s.c.) arteries also show hypersensitivity to NE and phenylephrine (Kurvers et al., 1998). In rats with SNL, venous concentrations of NE metabolites are decreased in the ipsilateral paw as compared to the contralateral paw at 2 weeks post-injury (Raja et al., 1995). Further, NE and other vasoconstrictors, such as vasopressin and angiotensin II, have been shown to elicit an enhanced excitation of DRG (dorsal root ganglion) neurons in SNL rats that correlates with their vasoconstrictive effects (Häbler et al., 2000). Therefore, some animal models of neuropathic pain show vascular abnormalities that may contribute to pain mechanisms as is also thought to occur in CRPS.

Additional studies have documented both inflammatory and motor abnormalities in animal models of neuropathic pain. Thus, there is increased plasma protein extravasation in the rat hind paw skin after SNT (Kingery et al., 2001, 2002, 2003). Also, early after injury (1-4 days), there is an increase in paw volume, oedema and accumulation of polymorphonuclear leukocytes in muscle tissue of the CCI hind limbs (Daemen et al., 1998a; Gradl et al., 2004). There is also apoptosis in muscle between 4 and 14 days after CCI of the sciatic nerve, as indicated by increased caspase 3 protein levels and Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling (TUNEL) staining, and a decreased myofibrillar ATPase reaction (Daemen et al., 1998b; Gradl et al., 2004; Gradl et al., 2006). There are also later (21-60 days) bilateral degenerative changes in the proximal and distal motor nerve fibers in CCI rats, as revealed by a marked decrease of ACHsterase-positive fibers (Bullens et al., 1998). These findings may underlie motor dysfunction or trophic changes observed in these animals. Therefore, animal models of nerve injury show vascular and motor abnormalities that are similar to symptoms in patients with CRPS-II. Evidence also indicates that there may be a progression from hot to cold limbs in neuropathic rats, which depends on an initial reduction in SNS activity and a gradually developing vascular hypersensitivity to adrenergic transmitters.

1.4.2 Anti-sympathetic treatments in animal models of CRPS-II

While no studies have looked at the anti-hyperalgesic effects of anti-sympathetic treatments or α-adrenergic antagonists in animal models of CRPS-I, many studies have assessed their role in animal models of neuropathic pain or CRPS-II. Anti-sympathetic treatments include lumbar surgical sympathectomies, systemic guanethidine or 6-hydroxydopamine (6-OHDA) injections, and α-adrenergic antagonist treatments. Systemic administration of guanethidine is most related to regional blocks clinically, since it is generally thought not to cross the blood-brain barrier, and not to affect cerebrospinal fluid and brain tissue NE levels (Johnson & O'Brien, 1976; Peskind et al., 1986). In addition, α_1 and α_2 adrenergic antagonists have also been used in rats with neuropathic pain, as they have for CRPS patients.

Early studies assessed the effects of repeated guanethidine or 6-OHDA treatments which produce a functional (short term guanethidine treatment) or anatomical (6-OHDA), neonatal or long-term guanethidine treatment chemical sympathectomy. Wall et al. (1979) showed that repeated administration of guanethidine starting 4 days after injury, reduced autotomy in rats with sciatic and saphenous nerve transections. Guanethidine was also shown to reduce autotomy when administered prior to the same surgery in rats (Coderre et al., 1984). Pre- or post-injury administration of guanethidine also led to significant reductions in heat hyperalgesia and cold allodynia (and post-injury slightly reduced mechanical allodynia) in rats with CCI of the sciatic nerve (Neil et al., 1991). Perrot et al. (1993) found that 7 daily i.v. guanethidine post-treatments attenuated mechanical and cold hyperalgesia in CCI rats. In contrast, pre-injury guanethidine administration has been found to augment autotomy in CCI rats (Neil et al., 1991). Guanethidine administered a few days after established PSNL pathology decreased both mechanical allodynia and heat hyperalgesia in PSNL rats (Tracey et al., 1995). Similarly, guanethidine administered 10 days after PSNL injury in the mouse reduced thermal hyperalgesia and mechanical allodynia (Malmberg & Basbaum, 1998). Shir & Seltzer (1991) found that pre-emptive administration of guanethidine prevents heat hyperalgesia, but not mechanical allodynia, after

PSNL, while administration several months after surgery reverses both mechanical allodynia and heat hyperalgesia. In contrast, if guanethidine was administered during PSNL surgery, it actually worsened mechanical allodynia and heat hyperalgesia (Shir & Seltzer, 1991). Guanethidine also reduced mechanical allodynia when given 2 weeks after SNL (Kim et al., 1993). However, Lavand'homme et al. (1998) showed that guanethidine treatment during SNL surgery does not attenuate development of mechanical allodynia. Neonatal guanethidine sympathectomy attenuated cold allodynia, but had no effect on mechanical allodynia and hyperalgesia in SNI rats (Pertin et al., 2007). Chemical sympathectomy with 6-OHDA after injury reduced heat hyperalgesia, but not mechanical allodynia, in mice with tibial nerve transections (Kingery et al., 2000). However, neither mechanical allodynia nor mechanical hyperalgesia are significantly reduced when 6-OHDA-induced chemical sympathectomy is performed 1 week after SNL (Wei et al., 2002).

Several studies have also assessed the effects of surgical sympathectomy or anaesthetic blocks of the lumbar sympathetic ganglia in animal models of neuropathic pain. Surgical sympathectomy performed during the CCI surgery attenuated cold and heat hyperalgesia, but not mechanical hyperalgesia (DesMeules et al, 1995). Surgical sympathectomy performed prior to SNI attenuated cold allodynia, but had no effect on mechanical allodynia or hyperalgesia (Zhao et al., 2007). Surgical sympathectomy or anaesthetic blocks, of the lumbar sympathetic ganglia performed 4-8 days after injury significantly reduce mechanical and cold allodynia and heat hyperalgesia in SNL rats (Choi et al., 1994; Chung et al., 1996; Park et al., 2000). Surgical sympathectomy also alleviates heat hyperalgesia and mechanical allodynia when performed 7 days prior, as well as between 1 and 5 weeks after, SNL (Kim et al., 1993; Kinnman & Levine, 1995). However, Lavand'homme et al. (1998) showed that surgical sympathectomy during SNL surgery does not attenuate development of mechanical allodynia. Similarly, Ringkamp et al. (1999a) showed that surgical sympathectomy 1 or 3 weeks prior, does not reduce mechanical allodynia or mechanical hyperalgesia in SNL rats. Recently, Xie et al. (2001b) showed that the

more limited lumbar region 5 (L5) sympathectomy used by Ringkamp et al (1999a), reduced SNL-induced mechanical allodynia less than did the L5-L6 sympathectomy used by Kim et al. (1993). Lee et al. (2001) showed that surgical sympathectomy was more effective at reducing allodynia when mechanical hypersensitivity was measured in the toe area which displays more sensory abnormality in the SNL model, rather than in the plantar area of the rat foot. Further, it was more effective in rats that had lower mechanical thresholds after SNL surgery (Lee et al., 2001). Kim et al. (1997) compared the effectiveness of surgical sympathectomy 1 week after injury in the SNL, PSNL, and CCI models. They found greatest reductions for mechanical allodynia in SNL rats, some reduction in PSNL rats, and no reduction in CCI rats. In regards to cold allodynia, all three models showed significant reductions after sympathectomy, although the effect was greater again in the SNL model.

Systemic administration of α-adrenergic antagonists have also been found to reduce allodynia and hyperalgesia in various animal models of neuropathic pain. Phentolamine and prazosin, but not yohimbine, reduce cold allodynia in rats with neuropathy of the tail (Kim et al., 2005a; Kim et al., 2005b). Alternatively, the α_2-adrenergic antagonists yohimbine, atipamezole, and L659-066 all significantly reduce mechanical allodynia and heat hyperalgesia in mice with tibial nerve transactions. Systemic administration of either phentolamine, prazosin, or the α_2-adrenergic antagonist, SKF86466, reduce thermal hyperalgesia in CCI rats (Hord et al., 2001).Yohimbine, but not prazosin, has been found to reduce mechanical and heat hyperalgesia in PSNL rats (Tracey et al., 1995). However, the hyperalgesia produced by the PSNL is unaltered in mutant mice lacking the A, B, or C subtypes of α_2-adrenoceptors (Malmberg et al., 2001). Phentolamine at a dose of 4 mg/kg reduces mechanical allodynia for up 4 days in SNL rats (Kim et al., 1993). Similarly, Lee et al. (1999) found that 5 mg/kg of either phentolamine, or the α_1 adrenergic receptor antagonist terazosin, reduced mechanical allodynia in SNL rats. However, both Ringkamp et al. (1999b) and Park et al. (2000) were unable to replicate this result with 5 mg/kg phentolamine. These inconsistent results may be explained by strain differences in the sensitivity

of phentolamine. Thus, Lee et al. (1997) showed that the same 5 mg/kg dose of phentolamine exhibited varying degrees of effectiveness against SNL hyperalgesia in different rat strains, with Lewis rats showing the greatest anti-allodynic effects, followed by Sprague-Dawley, Wistar, and finally Fischer rats, which were completely insensitive. Nam et al. (2001) found that 4 mg/kg phentolamine significantly reduced cold, but not mechanical, allodynia in SNL Sprague-Dawley rats. Thus, the anti-allodynic effectiveness of α-adrenergic antagonists seems to be influenced by testing modality, nerve injury model, and strain of rats.

1.4.3 Evidence for abnormal sympathetic-sensory coupling in animal models of CRPS-II

Many investigators have studied how the sympathetic efferents interact with sensory nerves after nerve injury. The primary focus of these investigations has been nerve electrophysiological recordings and anatomical characterization of afferent and sympathetic fibers. A *de novo* interaction between sympathetic and sensory nerves after nerve injury is hypothesized to maintain pain and is often termed "sympathetic-sensory coupling". Electrophysiological and anatomical evidence for abnormal sympathetic-sensory coupling has been described after nerve injury both in primary afferent axons and DRG.

Wall & Gutnick (1974) performed the landmark study that showed evidence of *de novo* adrenergic sensitivity in regenerating primary afferent axons after nerve injury. Recording from neuromas of rats with SNT, it was seen that afferent fibers showed increased activity after application of NE, an effect blocked by the α-adrenergic antagonist phentolamine. Although epinephrine, which binds both to α and β-adrenergic receptors, also induced excitation, the β-adrenergic agonist isoprenaline, had no effect, suggesting the activity was at α-adrenoceptors. Devor & Jänig (1981) demonstrated a similar result in a rat sciatic nerve neuroma in which afferent fibers were activated by both lumbar sympathetic stimulation and adrenaline. Discharges were blocked by phentolamine, but not the β-adrenergic antagonist propanolol. Lumbar sympathetic stimulation excited 26% of spontaneously active axons in a neuroma

of the cat peroneal nerve, while 39% were excited by systemic NE or epinephrine (Blumberg & Jänig, 1984). Approximately 60% of spontaneously active Aβ and Aδ fibers in a rat sciatic nerve neuroma responded to systemic epinephrine, and this effect was blocked best by the α_2-adrenergic antagonist yohimbine, and to a lesser degree by the α_1-adrenergic antagonist prazosin (Chen et al., 1996). Therefore, the evidence suggests that after nerve injury, a significant proportion of regenerating sensory afferent fibers respond abnormally to sympathetic activation, and this involves primarily α-adrenergic receptors. Interestingly, however, neonatal chemical sympathectomy or adult surgical sympathectomy also increases NE sensitivity in spontaneously active afferent fibers in sciatic nerve neuromas (Rubin et al., 1997).

There is also evidence of *de novo* sympathetic-sensory coupling in the distal endings of injured primary afferent fibers. After lesion of the auricular nerve, both sympathetic stimulation and local administration of NE or epinephrine resulted in excitation in a subset (~20%-33%) of cutaneous C-fibers in the rabbit ear. α_2-adrenergic antagonists, but not α_1-adrenergic antagonists, blocked the activity, and no fibers were excited by sympathetic stimulation in animals without nerve injury (Sato & Perl, 1991; O'Halloran & Perl, 1997). Also in this model, sympathetic activation of distal afferents appeared to be specific to C-fibers (Bossut & Perl, 1995). Approximately 60% of distal C-fibers are excited by phenylephrine and ~33% are excited by the α_2-adrenergic agonist UK-14304 in monkeys with SNL (Ali et al., 1999). However, close-arterial NE injection induced C-fiber excitation in only ~12% of nociceptors after SNL in rats (Nam et al., 2000). Here, prazosin, and not yohimbine, blocked the NE-induced excitation of nociceptors. Also, in addition to C-fibers, some A-δ fibers were excited by intra-arterial NE, in contrast to the monkey and rabbit studies. Therefore, after nerve injury a certain proportion of nociceptive afferents are likely to respond abnormally to sympathetic activity, but the class of activated nociceptor may not necessarily be the same in different species.

Several studies have also characterized adrenergic sensitivity in the DRG of nerve-injured animals. Burchiel (1984) first reported that ~48 % of rat DRG

neurons are excited by epinephrine, immediately after SNT, and 60% were excited 1 week after transection. This effect was blocked by phentolamine, and not produced by the β-adrenergic agonist isoprenaline. Devor et al. (1994) later showed that stimulation of the sympathetic chain excited ~62% of spontaneously active DRG neurons after SNT, compared to only 2% in control animals. Phentolamine again blocked the excitation. After CCI, DRG neurons are excited by both NE and sympathetic stimulation. This was seen in ~80-90% of C-fibers and ~50% of A-δ fibers, and the excitation was blocked by yohimbine, but not prazosin (Xie et et al., 1995b). DRG cells recorded *ex vivo* after CCI were also dose-dependently excited by NE (Petersen et al., 1996; Zhang et al., 1997). After both axotomy and CCI, NE has been shown to increase cell excitability in isolated DRG cells, by acting on α_2-adrenergic receptors and causing a decrease in potassium outflow (Abdullah & Smith, 1997; Honma et al., 1999). In SNL rats, 22% of DRG neurons also showed hypersensitivity to sympathetic stimulation, and the effect is blocked by yohimbine, but not prazosin (Leem et al., 1997). It has further been shown that DRG neuron hyperexcitability to sympathetic stimulation after SNT changes over time, with predominant hyperexcitability between 4-22 days, mixed responses between 60-93 days, and primarily suppressive responses 110-171 days post-transection (Michaelis et al., 1996). Occasional suppression of DRG activity by adrenergic stimulation has been confirmed after SNT in other studies (Devor et al., 1994), although the mechanisms are not well understood. Nevertheless, these findings suggest that abnormal sympathetic-sensory coupling may occur in the DRG in various types of nerve injury models, although the type and duration of nerve injury may be important.

In addition to activation of sensory afferent by sympathetic stimulation, there are also changes in the expression of α-adrenergic receptor subtypes in DRG after nerve injury. Thus, there is an increase in α_{2a}-adrenergic receptor mRNA, and a decrease in α_{2c} mRNA, after SNT (Shi et al., 2000). A similar increase is seen for DRG α_2-adrenergic receptors after rat SNL (Cho et al., 1997). There is also an increase in α_{1b} receptors, and a decrease in α_{1a}, in DRG after

SNL (Xie et al., 2001a). The existence of multiple subtypes and their differential changes with nerve injury may complicate interpretation. Nevertheless, these results are consistent with the suggestion that both upregulated α_1- and α_2-adrenergic receptors may be involved in abnormal sympathetic-sensory interactions after nerve injury.

Another line of evidence suggestive of abnormal interaction between sympathetic efferents and sensory afferents after nerve injury comes from demonstrations of anatomical changes in sympathetic neurons. McLachlan et al. (1993) and Chung et al. (1993) first published reports of noradrenergic fiber sprouting after both SNT and SNL. Sympathetic noradrenergic fibers were shown to form basket like structures around neuronal somata in the DRG three weeks after SNT. This sprouting correlated with the time that sympathetic stimulation evoked excitation of primary sensory neurons in their studies. Increases in sympathetic-immunolabeled fibers were detected in the spinal nerve and around some DRG cells within 2 days after SNL. Surgical sympathectomy 4 days post-injury reversed sympathetic fiber sprouting, mechanical hypersensitivity and cold allodynia in SNL rats (Chung et al., 1996). Sprouting of sympathetic fibers to the DRG is detectable at 4 days after CCI injury (Ramer & Bisby, 1997). Sprouting is further detectable at the glabrous skin starting at 2 weeks post-injury and peaking between 4 to 6 weeks in CCI rats (Yen et al., 2006). Ramer et al. (1998) proposed that different types of sympathetic fiber sprouting could occur, such as collateral sprouting of non-injured fibers distal to the nerve lesion, as well as regenerative sprouting of injured fibers. This may in turn explain differences in sprouting patterns between animal models. Sprouting fibers are also known to form branches far from the injury site (Chung & Chung, 2001). Chronic spinal nerve growth factor (NGF) administration also induces sympathetic nerve sprouting to sensory ganglia in uninjured animals, and results in the excitation of *ex vivo* DRG neurons to applied NE (Jones et al., 1999). In contrast, anti-NGF treatments reduce sprouting and basket formation in SNL rats (Deng et al., 2000). Recent evidence has also shown that continuous systemic lidocaine during surgery, or topical lidocaine 7 days after surgery, can significantly reduce sympathetic fiber

sprouting and sympathetic activation of primary afferent neurons after spinal nerve transection (Zhang et al., 2004; Xie et al., 2007). Therefore, it appears that sympathetic sprouting is prominent after nerve injury and may contribute to abnormal sympathetic-sensory coupling in animal models of neuropathic pain.

Lee et al. (1998) performed a comparison of the amount of sympathetic fiber sprouting after SNL, CCI, and SNT and found significant sprouting in all three models. However, the time course was different with the SNL showing significantly earlier sprouting than the other models. Neither after SNL nor CCI was there a good correlation of sprouting with sensory symptoms, although a better correlation was observed after SNT. A lack of correlation of sensory symptoms with anatomical changes has also been seen after rat tail nerve injury. Although behavioural signs were equivalent after two different nerve lesions, cutting caudal nerves innervating the tail closer to the DRG resulted in more extensive sprouting than cutting nerves further away from the DRG (Kim et al., 1996). Also animals that developed strong sensory abnormalities showed equivalent sprouting to those that did not develop prominent neuropathic pain behaviours (Kim et al., 1999a). Whether the animals responded with anti-allodynia to phentolamine treatment also did not correlate with the amount of sprouting (Kim et al., 1998). Also, more severe nerve injury produced by cutting a greater number of nerves resulted in more sprouting, but did not produce greater mechanical allodynia, cold allodynia, or heat hyperalgesia (Kim et al., 2001). Hence, it seems that sympathetic fiber sprouting is clearly a consequence of nerve injury, but the link of sprouting to nociception is tenuous.

A small number of studies have also attempted to replicate, in animal models of neuropathic pain, the clinical demonstrations of NE-evoked pain in CRPS patients. Further animal studies have assessed the rekindling of CRPS-like pain, as seen in patients whose pain is relieved by sympathectomy or sympathetic blocks. Intraplantar (i.pl.) injection of NE has been found to enhance both mechanical hyperalgesia and heat hyperalgesia in PSNL rats (Tracey et al., 1995), although lower doses of NE did not enhance mechanical allodynia in SNL rats (Moon et al., 1999). Rekindling was not observed by Tracey et al. (1995), since

intracutaneous NE did not restore the mechanical and heat hyperalgesia that were relieved following treatment with guanethidine. However, rekindling was demonstrated when i.pl. NE restored mechanical allodynia in PSNL rats that had relief following surgical sympathectomy (Xie et al., 1995a). A role for α_2 receptors was implicated, since the re-kindling was blocked by the α_2-adrenergic antagonist izadoxan, but not the α_1-adrenergic antagonist terazosin.

1.4.4 Animal models of CRPS-I

While the traumatic surgical nerve injury models of CRPS-II have been well characterized, there have only been a few attempts to model CRPS-I, the key being to cause neuropathic-like symptomatology without initiating major nerve injury. Vatine et al. (1998) described one of the first novel animal models of CRPS-I produced by electrically stimulating the sciatic nerve supramaximally (0.5 Hz, 8 mA) for 10 minutes. The protocol presumably led to activation of both A and C fibers, and resulted in thermal hyperalgesia that lasted for up to three weeks after stimulation, with a lesser degree of cold and mechanical allodynia. The sensory symptoms were hypothesized to be due to sensitization of the nerve or its central connections, and not inflammation or nerve injury. While this is an interesting model, it was not followed up except to put into context with other work (Vatine et al., 2001). Other non-painful symptoms associated with CRPS-I were not observed in this model.

Another animal model was developed by van der Laan et al. (1997a; 1997b; 1998a) which involved infusing the free-radical donor, tert-butyl-hydroperoxide, in the femoral artery of awake rats continuously for 24 hours. Signs of inflammation such as redness, paw swelling, increased skin temperature, and spontaneous pain behaviours were seen both during infusion and at 24 hours, subsiding within a couple of days. Rats also showed pain behaviours when placed on a hot plate one-week post-injury, plasma extravasation up to 7 days post-injury, and bilateral mechanical hypersensitivity that lasted up to 4 weeks. These effects were significantly attenuated with systemic administration of a free-radical scavenger before and after free radical donor infusion. While this model

represents an interesting attempt to model CRPS-I, this method was not followed up with any further studies.

Gradl et al. (2005) showed that controlled-impact soft tissue injury, femoral intra-arterial infusion of inflammatory mediators contained within the supernatant of traumatized muscle, or the combination of both, can induce signs of inflammation (swelling, leukocyte activation), ischemia (as measured by reduced functional capillary density) and mechanical allodynia from 24 hours to 14 days post-injury (Gradl et al., 2006). Since such inflammatory/ischemic pathology was not observed in CCI rats, it was suggested that the animal model was more relevant to CRPS-I. Gradl et al (2007) also demonstrated that intra-arterial substance P infusion for 24 hours in rats can further be used to mimic CRPS-I. Animals show mechanical allodynia, local muscle inflammatory responses, and oedema. No evidence of nerve injury or apoptotic muscle cells was noted in the hind limb skeletal extensor muscles after chronic substance P infusion. This is also different from CCI, which shows evidence of apoptosis, but an absence of inflammation, in these muscles (Gradl et al., 2004; Gradl et al., 2006). While this model is interesting, behavioural observations were only followed for 4 days post-procedure. Interestingly, they did not find evidence of spontaneous pain behaviours or thermal hyperalgesia.

A novel animal model was recently created by inducing a minor nerve injury with needle puncture of the tibial or sural nerves (Siegel et al., 2007). Between 50-80% of rats developed mechanical allodynia that lasted 7 days, and between 30-50% still had mechanical allodynia at 14 days post-injury. There was a small incidence of mechanical hyperalgesia and cold allodynia (10-30%). There was no significant difference between tibial or sural nerve injury, and no correlation between needle size used for needle stick and sensory changes. Although the largest gauge needle produced more postural abnormalities, there was no more than 50% axon degeneration. This model complements the results of Oaklander et al. (2006) which showed evidence of minor nerve injury in CRPS-I patients. This model remains to be characterized further, but suggests that minor nerve injury may induce CRPS symptomatology.

Another particularly interesting animal model for CRPS-I was developed by Guo et al. (2004), and involves rat tibial fracture and casting for 4 weeks. Up to 44% of CRPS cases may be as a result of fractures (De Mos et al., 2006), and up to 31% of distal tibial fractures result in CRPS-I (Sarangi et al., 1993). This makes the animal model by Guo et al. (2004) particularly relevant to the clinical picture of CRPS-I. Animals developed a variety of signs and symptoms resembling CRPS-I after fracture and casting, such as hyperthermia, oedema, mechanical allodynia, increased cytokines in hind paw skin and nerve, and a reduction in bone mineral density. Mechanical allodynia lasted at least 16 weeks, along with the oedema and hyperthermia, and it was reversed with a neurokinin-1 (NK-1) antagonist and soluble TNF receptor. Glucocorticoid administration attenuated oedema, protein extravasation, and hyperthermia, but not mechanical allodynia. Further, i.v. substance P administration leads to an enhanced neurogenic inflammatory response in this model (Guo et al., 2006; Sabsovich et al., 2008b). This last finding parallels the findings by Leis et al. (2003) in CRPS patients described above.

1.5 The vascular functions of the sympathetic nervous system

1.5.1 Overview of the sympathetic system and adrenergic receptors

The SNS, along with the parasympathetic nervous system (PSNS), and sometimes the enteric nervous system, are classically described as part of the autonomic nervous system. The autonomic nervous system comprises the nerves that innervate various tissues, blood vessels and organs in the vertebrate body. Divisions of the SNS and PSNS are made on anatomical criteria with the PSNS providing innervations mainly from the cranial and sacral region of the spinal cord, while the SNS provides innervations mainly from the thoracic and lumbar regions of the spinal cord (Langley, 1903). In the SNS, efferent neurons from the spinal cord termed “pre-ganglionic neurons” run to several ganglia (such as superior cervical ganglion, stellate ganglion, mesenteric ganglion) synapsing with “post-ganglionic neurons”. Ganglia are often interconnected by nerve trunks and some form plexuses far away from target organs, and post-ganglionic neurons often are long and branch out ultimately involving thousands of varicosities that

that release neurotransmitters to the target organ. At the ganglia, the major neurotransmitter of the sympathetic pre-ganglionic neuron is ACH, while at the terminal of the sympathetic post-ganglionic neuron (SPGN), it is NE (Jänig, 2006a). Arteries, arterioles, and some veins are heavily innervated by sympathetic noradrenergic neurons. Sympathetic activation from the thoracic and lumbar spinal cord regions results in the release of NE in muscle and skin resulting in vasoconstriction by action mainly on α-adrenergic receptors (Jänig, 2006b).

Adrenergic receptors were one of the first receptor families to be identified and heavily characterized. They are all G protein-coupled receptors (GPCRs), and the elucidation of their distinct subtypes and signalling pathways has served as prototypes for other GPCRs later discovered. Three α_1-adrenergic receptors (α_{1a}, α_{1b}, and α_{1d}) have been discovered (Hieble et al., 1995), all of which act through a $G_{q/11}$ protein to increase intracellular calcium. Some of the major synthetic α_1 adrenergic agonists are phenylephrine and cirazoline, while the major antagonists are prazosin and terazosin. There are also three important α_2-adrenergic receptors (α_{2a}, α_{2b}, and α_{2c}) (Bylund et al., 1994), which act through a G_i protein to decrease cyclic adenylate monophosphate (cAMP). Major synthetic agonists for these receptors include clonidine and UK14304 and major antagonists include yohimbine and chlorpromazine. Further, there are also three β-adrenergic receptors (β_1, β_2, and β_3) (beyond the scope of this thesis) that primarily act by coupling to a G_s protein to increase cAMP. The major endogenous ligand for β-adrenergic receptors is adrenaline or epinephrine, while for the α-adrenergic receptors, it is noradrenaline or NE (Summers & McMartin, 1993).

The different adrenergic receptor subtypes are widely distributed in the body to different extents in various tissues and vary between species. Activation of each receptor alone or with other receptors depends on ligand selectivity and expression of specific receptor subtypes (Minneman, 2006). Both α_1- and α_2-adrenergic receptors are found throughout the central and peripheral nervous system. In the peripheral tissues, some of the prominent locations identified are the blood vessels, liver, pancreas, prostate, penis, urethra, kidney, bladder, and immune cells (Perez et al., 2006). Adrenergic receptors are also widely distributed

in brain areas including the reticular formation, periaqueductal gray, rostro-ventromedial medulla, thalamus, and amygdala. Discrete brain microinjections and intracerebroventricular infusion of adrenergic compounds has been long known to modulate nociception (Schmitt et al., 1974; Sagen & Proudfit, 1985; Pertovaara, 2006). Various descending modulation circuits from the brain to spinal cord contain noradrenergic fibers, with their activation resulting in spinal NE release (Sagen & Proudfit, 1987). It has also long been known that spinal injection of α-adrenergic agonists such as NE and clonidine can modulate nociceptive processes and alleviate hyperalgesia in animal models of chronic pain (Headley et al., 1978; Reddy & Yaksh, 1980; Yaksh et al., 1995). All α-adrenergic receptor subtypes have been detected in the spinal cord, with a particular prominence of the α_{1a}, α_{1d}, and α_{2a} subtypes (Pieribone et al., 1994; Day et al., 1997; Stone et al., 1998).

After nerve injury, the spinal distribution of α-adrenoceptors is known to change, and the α_2-adrenergic receptors are generally increased (Stone et al., 1999; Shi et al., 1999). This may be related to the sometimes reported increased potency of intrathecal α_2 agonists in neuropathic pain states (Eisenach et al., 1996; Bantel et al., 2005). All α-adrenergic receptors are also expressed in the DRG, with a particular prominence for the α_{1a}, α_{2a}, and α_{2c} (Gold et al., 1997; Xie et al., 2001a; Nicholson et al., 2005). With nerve injury, the distribution of subtypes in DRG also changes, although the pattern differs from the spinal cord, as has been demonstrated for both α_1- and α_2-adrenergic receptors (Cho et al., 1997; Shi et al., 2000; Xie et al., 2001a). Therefore, adrenergic receptors both centrally and peripherally are predicted to be involved chronic pain states. Studies using adrenergic receptor drugs in pain states have been limited due to lack of subtype-selective pharmacological agents, as well as lack of studies assessing the functional implications of the variable distribution of subtypes in different tissues and species.

1.5.2 Vascular actions of adrenergic drugs

The doses of adrenergic agents that have been used in CRPS patients are usually demonstrated to induce vasoactive effects. In addition to the drug used,

the route of administration is particularly important for determining ultimate function of α-adrenergic drugs. For example, topical administration of either α_2-adrenergic agonists (Davis et al. 1991), or systemic administration of α_1- or α_2-adrenergic antagonists produces analgesia in CRPS patients (Abram & Lightfoot, 1981; Ghostine et al., 1984; Raja et al., 1991; Stevens et al., 1993; Muizelaar et al., 1997). Not only the presence of adrenergic subtypes in different tissue, but also whether receptors are predominantly presynaptic or post-synaptic is important in determining function. While α-adrenergic agonists, such as NE, constrict blood vessels and α-adrenergic antagonists inhibit this, it has long been known that α-adrenergic antagonists such as phenoxybenzamine or yohimbine can also cause increases in NE release (Brown & Gillespie, 1957; Starke et al., 1971; Starke, 1972, Starke et al., 1975a; Starke, 1987). With the identification of the α_1- and α_2-adrenergic receptor subtypes, Langer (1980) proposed that adrenergic receptors on blood vessels were mainly α_1-adrenergic receptors, and receptors on SPGNs were mostly α_2-adrenergic receptors. Since then, both α_1- and α_2-adrenergic receptors have been identified on blood vessels and SPGN, as well as pre- and post-synaptically in noradrenergic synapses in the CNS (Brock, 1995). Systemic α_2-adrenergic receptor drugs usually have effects more consistent with a more important action on the SPGN or presynaptically. An important role for this mechanism was first demonstrated with administration of the α_2-adrenergic antagonist, idazoxan, using *in vivo* microdialysis in the rat cortex where the drug enhanced NE release (Dennis et al., 1987). In human volunteers, systemic administration of yohimbine has been shown to increase sympathetic activity as measured by heart rate, blood pressure, and has increased the rate of appearance of enhanced arterial and limb NE plasma levels (Grossman et al., 1991; Hedner et al., 1992). Systemically-administered clonidine, on the other hand, decreases sympathetic activity as measured by blood pressure, sympathetic nerve activity, and plasma NE levels (Esler et al., 1985; Muzi et al., 1992). Mutant animals lacking α_2-adrenergic receptors lack the normal inhibition

of NE release by selective α_2-adrenergic agonists in various tissues, including at SPGNs (Starke, 2001; Trendelenburg et al., 2003).

α-adrenergic receptors on vascular smooth muscle cells are also well known to be vasoactive and elicit contraction in pulmonary arteries (Starke et al., 1975b). α_2-adrenergic activation has also been shown in vascular smooth muscle (Drew & Whiting, 1979; Timmermans & Van Zwieten, 1981). Madjar et al., (1980) found that yohimbine-induced potentiation of the vasoconstriction induced by sympathetic stimulation in the rabbit hind limb was much less than in the pulmonary arteries, and hypothesized that it must be due to an additional opposing action of yohimbine at α_2-adrenergic receptors in hind limb vascular smooth muscle cells. Gardiner & Peters (1982) also found that yohimbine's action at hind limb blood vessels blocked NE and sympathetic stimulation-induced vasoconstriction in cats and dogs. Comparisons of a series of α_2-adrenergic drugs with different potencies also yielded evidence that NE vasoconstrictive effects involve α_2-adrenergic receptors on smooth muscle cells (McGrath, 1982). Although, less strong than α_1 mediated vasoconstriction, studies *in vivo* in the pithed rat model, or with vascular bed autoperfusion, have demonstrated significant vasoconstriction with α_2-adrenergic agonists (Yamamoto et al., 1984; Pedrinelli and Tarazi, 1985). Locally-administered α_2-adrenergic agents can also have different vasoactive effects at lower and higher concentrations (Hermann et al., 2005). Hence, local administration of α_2-adrenergic agents is likely to also act on both at the SPGN and at receptors on smooth muscle cells.

It is known that α-adrenergic vasoaction can differ between vascular beds. Medgett & Ruffolo (1988) demonstrated that neurogenic vasoconstriction induced by sympathetic stimulation was reduced by α_1- and α_2-adrenergic antagonists in the saphenous artery or cutaneous vascular beds, but only by α_1-adrenergic antagonists in the femoral artery or skeletal muscle. This may be related to older studies which show that NE displays stronger vasoconstriction in more superficial tissues than in the deep tissues (Abboud & Eckstein, 1968). Interestingly, in the cremaster muscle, NE-induced vasoconstriction in large arterioles is antagonized

by both α_1- and α_2-adrenergic antagonists, while in the small pre-capillary arterioles, α_2-adrenergic antagonists, but not α_1-adrenergic antagonists, block NE-induced vasoconstriction (Faber, 1988). However, in the same model, sympathetic stimulation-induced vasoconstriction in large arterioles is antagonized by only α_1-adrenergic antagonists, while only α_2-adrenergic antagonists block vasoconstriction in the small pre-capillary arterioles (Ohyanagi et al., 1991). Various studies have also demonstrated that veins show different distributions of adrenoceptors than arteries, with α_2-adrenergic receptors suggested to be closer to the sympathetic nerve terminals (Guimaraes & Moura, 2001). Further, using subtype-selective α_1- and α_2-adrenergic drugs, venule and arteriole vasoconstrictive potency differs in the cremaster muscle, although both α_1- and α_2–adrenergic subtypes are involved in each (Leech & Faber, 1996). While an α_{1a}-adrenergic antagonist most effectively blocks NE-induced vasoconstriction in the autoperfused rat hind limb (Zhu et al., 1997), and in rat femoral resistance arteries (Jarajapu et al., 2001b), sympathetic stimulation-induced vasoconstriction is also blocked by α_{1d}-adrenergic antagonists (Zacharia et al., 2004). Studies with knockout animals have confirmed involvement of the α_{1a}- and α_{1d}-adrenergic receptors in vasopressor responses, however there is also evidence of the α_{2a}- and α_{2b}-adrenergic receptors in blood pressure regulation and α_{2c}-adrenergic receptor effects on catecholamine release (Simpson, 2006; Tan & Limbird, 2006). Hence, in vascular tissues, various α-adrenergic drugs may act at differing receptors on both vascular smooth muscle cells and the SPGN, although the question of attributing vasoactive properties to particular to adrenergic receptor subtypes still remains to be elucidated and depends on the tissue type.

Statement of Purpose

CRPS-I is a poorly understood and devastating chronic pain disorder often failing treatment. While research on neuropathic pain has benefited from many well-characterized animal models using injury of major peripheral nerves, understanding of CRPS-I processes has been limited by a lack of suitable and

well-characterized animal model. With an increasing number of studies in the recent years showing that ischemia and vascular abnormalities are present in limbs of patients with CRPS-I, the first aim of this thesis was to determine whether an I-R injury would induce signs of CRPS-I, and result in robust and quantifiable nociception in addition to vascular injury.

Traditionally, CRPS-I is thought to involve primarily SNS abnormality, and some CRPS patients obtain relief from sympathetic blockade. Surgical and chemical sympathectomy has also been reported to relieve sensory abnormalities in rodents with nerve injury. Hypersensitivity to adrenergic agents, likely due to abnormal sympathetic-sensory coupling, has further been demonstrated in animal models of neuropathic pain. Therefore, the second aim of the thesis was to test whether anti-sympathetic treatment relieves mechanical allodynia in rats with CPIP. Since anti-sympathetic procedures result in warming of the limb and vasodilatation, we also examined whether enhanced vasodilatation could be a mechanism of pain relief in CPIP rats.

The third aim of this thesis was to examine whether CPIP rats showed exaggerated nociceptive behaviours and vascular vasoconstrictive hypersensitivity to NE. Both phenomena have been reported in CRPS, particularly for those patients that show pain relief to anti-sympathetic treatments. Therefore, NE was injected intradermally in CPIP rats and nociceptive behaviours were quantified. We also examined whether painful responses to NE could be relieved by α_1- and α_2-adrenergic antagonists, as well as a vasodilator, or whether they could be mimicked by intradermal injection of non-adrenergic vasoconstrictors or an eNOS inhibitor. Parallel experiments were performed in anaesthetized animals to examine whether CPIP rats with mechanical allodynia display greater vascular hypersensitivity to NE as measured by changes in hind paw blood flow using laser Doppler flowmetry. The overall aim was to establish whether enhanced vasoconstrictor reactivity and chronic tissue ischemia may be associated with mechanical allodynia in a novel animal model of CRPS-I.

Chapter 2

Chronic post-ischemia pain (CPIP): a novel animal model of complex regional pain syndrome-Type I (CRPS-I; reflex sympathetic dystrophy) produced by prolonged hindpaw ischemia and reperfusion in the rat

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2.1 Abstract

A neuropathic-like pain syndrome was produced in rats following prolonged hindpaw ischemia and reperfusion, creating an animal model of complex regional pain syndrome-Type I (CRPS-I; reflex sympathetic dystrophy) that we call chronic post-ischemia pain (CPIP). The method involves placing a tourniquet (a tight fitting O-ring) on one hindlimb of an anesthetized rat just proximal to the ankle joint for 3 h, and removing it to allow reperfusion prior to termination of the anesthesia. Rats exhibit hyperaemia and oedema/plasma extravasation of the ischemic hindpaw for a period of 2–4 h after reperfusion. Hyperalgesia to noxious mechanical stimulation (pin prick) and cold (acetone exposure), as well as mechanical allodynia to innocuous mechanical stimulation (von Frey hairs), are evident in the affected hindpaw as early as 8 h after reperfusion, and extend for at least 4 weeks in approximately 70% of the rats. The rats also exhibit spontaneous pain behaviors (hindpaw shaking, licking and favoring), and spread of hyperalgesia/allodynia to the uninjured contralateral hindpaw. Light-microscopic examination of the tibial nerve taken from the region just proximal to the tourniquet reveals no signs of nerve damage. Consistent with the hypothesis that the generation of free radicals may be partly responsible for CRPS-I and CPIP, two free radical scavengers, NAC and 4-hydroxy-2,2,6,6-tetramethylpiperydine-1-oxyl (TEMPOL), were able to reduce signs of mechanical allodynia in this model.

Keywords: CRPS-Type I; Chronic post-ischemia pain; Sympathetic dystrophy

2.2 Introduction

Growing evidence suggests that complex region pain syndrome-Type I (CRPS-I, RSD) may depend in part on tissue ischemia. Skin capillary hemoglobin oxygenation (HbO_2) is lower (Koban et al., 2003), and skin lactate is increased, reflecting enhanced anaerobic glycolysis (Birklein et al., 2000a; Birklein et al., 2000b) in CRPS-I limbs. Also, cold CRPS-I limbs have impaired nutritive skin blood flow (Kurvers et al., 1995). Muscle tissue in amputated CRPS-I limbs was found to exhibit lipofuscin pigment, atrophic fibers, and severely thickened basal membrane layers of the capillaries, consistent with oxidative stress and ischemic conditions resulting from microangiopathy in muscle tissue (van der Laan et al., 1998b). There is also an impairment of high-energy phosphate metabolism in muscle tissue of CRSP-I limbs (Goris, 1998; Heerschap et al., 1993), suggestive of lowered mitochondrial oxygen supply.

Goris (1998) argued that CRPS-I depends on an exaggerated inflammatory response. Thus, there is increased density of perfused vessels, higher capillary filtration capacity (an index of microvascular permeability), and plasma extravasation in the affected limb in the early stages of CRPS-I (Matsumura et al., 1996; Oyen et al., 1993; Schürmann et al., 2001b). While these changes are accompanied by high arterial blood flow, there is an elevated peripheral venous pressure, and arteriovenous shunting in the affected limb of CRPS-I patients (Matsumura et al., 1996; Schürmann et al., 2001b). Thus, there is high arterial flow to the CRPS-I limb, but low oxygen consumption, as well as high lactate flux-indicative of tissue ischemia (Goris, 1991; Goris, 1998). CRPS-I may depend on ischemia–reperfusion (IR) injury which also produces arteriovenous shunting (Kennedy et al., 1981), and is known to contribute to ischemic contracture and compartment syndrome in traumatic (Hoover and Siefert, 2000) or tourniquet (Blaisdell, 2002) shock.

Previous studies in rats show that transient (5–12 min) tourniquet-induced tail IR causes hyperalgesia lasting at least 2 h (Gelgor et al., 1986; Vidulich and Mitchell, 2000). Prolonged (2 h) tourniquet-induced IR of the rat hindpaw produces an immediate hyperaemia on reperfusion, and subsequent persistent

hindpaw oedema (Somogyi and Selye, 1969). We examine here whether prolonged (3 h) IR of the rat hindpaw produces inflammatory and pain symptoms similar to CRPS-I in humans.

Considerable evidence suggests that oxygen free radicals may contribute to IR injury and possibly also CRPS-I. Hindlimb IR increases various free radicals in postcapillary venules (Blaisdell, 2002; Yassin, 1997). Free radical scavengers reduce heat-hyperalgesia in rats with CCI of the sciatic nerve (Khalil and Khodr, 2001; Khalil et al., 1999; Tal, 1996). CRPS-I symptoms are relieved following treatment with free radical scavengers (Geertzen et al., 1994; Goris, 1985; Goris, 1998; Goris et al., 1987; Perez et al., 2003; Zuurmond et al., 1996), and the incidence of CRPS-I after wrist fractures may be reduced by pre-emptive treatment with the anti-oxidant vitamin C (Amadio, 2000; Cazeneuve et al., 2002; De Lange-de Klerk, 2000; Zollinger et al., 1999). Thus, another purpose of this study was to examine the potential anti-hyperalgesic effects of free radical scavengers in our animal model of CRPS-I.

2.3 Materials and methods

2.3.1 Animals

The present studies employed male Long Evans hooded rats (275–325 g, Charles River, Québec, Canada). Rats were housed in groups of 3–4, with food and water available ad libitum, on a 12:12 h light: dark cycle. All treatments and testing procedures were approved by the Animal Care Committee at McGill University, and conformed to the ethical guidelines of the Canadian Council on Animal Care and the IASP (Zimmermann, 1983).

2.3.2 Hindpaw ischemia and reperfusion

CPIP was generated following exposure to prolonged hindpaw ischemia and reperfusion. Rats were anesthetized over a 3 h period with a bolus (40 mg/kg, i.p.) and chronic i.p. infusion of sodium pentobarbital for 2 h (13 mg/h for first hour, 6.5 mg/h for second hour). After induction of anesthesia, a Nitrile 70 Durometer O-ring (O-rings West, Seattle, WA, USA) with 7/32 in. internal diameter was placed around the rat's left hindlimb just proximal to the ankle joint. The O-rings were selected to produce a tight-fit that produced ischemia similar to

that produced by a blood pressure cuff inflated to 350 mmHg, and were left on the limb for 3 h. We standardized the position of the O-ring to a point on the limb just proximal to the medial malleolus. The application was standardized by sliding the O-ring off the outside of a 3 cm^3 syringe (that was cut in half), after the hindpaw was inserted into the syringe barrel as far as possible. The termination of sodium pentobarbital anesthesia was timed so that rats recovered fully within 30–60 min following reperfusion, which occurred immediately after removal of the O-ring. Sham rats received exactly the same treatment, except that the O-ring was cut so that it only loosely surrounded the ankle, and did not occlude blood flow to the hindpaw.

2.3.3 Hyperaemia and plasma extravasation

Hyperemia was examined by measuring the temperature of the plantar surface of the hindpaws using a thermocouple probe connected to a transducer (BAT-12, Physitemp, Clifton, NJ, USA). A temperature measurement was based on an average of three replicate recordings taken at various time points between 5 min and 4 h after reperfusion. Measurements were obtained from separate groups of CPIP (N=6) and sham (N=6) rats, and a hyperemia score for each animal was generated by subtracting the temperature measurement of the contralateral hindpaw from that of the ipsilateral hindpaw.

Oedema was assessed by determining the degree of plasma extravasation using a spectrophotometric analysis of Evans Blue dye extravasation from the ipsilateral, as compared to the contralateral, hindpaw (Yashpal & Coderre, 1998). Rats were briefly anesthetized with halothane (4%) and given an i.v. (tail vein) injection of Evans Blue dye (50 mg/kg in 2.5 ml/kg) 30 min prior to the desired measurement time. Thus, at 2, 12 or 24 h after reperfusion (N=14, 6, 16 for the three time points), or 2 h after sham treatment (N=6), rats were re-anesthetized with sodium pentobarbital (100 mg/kg, i.p.), and received an intracardiac perfusion with 0.9% saline to flush blood from the circulation. The ipsilateral and contralateral hindpaws were then removed by amputation at the ankle joint. The hindpaws were next incubated in 4 ml of formamide at 70 °C for 24 h to extract Evans Blue dye from the tissue. After cooling to room temperature, the amount of

extravasated dye from each hindpaw was determined by spectrophotometric measurement of absorbance at a wavelength of 655 nm and interpolation from a linear standard curve, and an oedema score for each animal was generated by subtracting the amount of extravasated Evans blue dye of the contralateral hindpaw from that of the ipsilateral hindpaw.

2.3.4 Mechanical and thermal sensitivity

The plantar surface of the ipsilateral and contralateral hindpaws of CPIP (*N*=15) and sham (*N*=10) rats was tested for mechano-allodynia, mechano-hyperalgesia, cold-allodynia and heat-hyperalgesia (in that order) over a period between 8 h and 4 weeks after hindpaw IR. Measurements of mechano-allodynia preceded cold-allodynia in order to avoid cold-induced reductions in mechanical thresholds (Kauppila, 2000). Rats that did not exhibit positive sensory symptoms (i.e. hyperalgesia or allodynia) by the 48 h measurement were excluded from the data presented here; approximately 70% of rats showed positive sensory symptoms. Pilot experiments with Sprague–Dawley rats (Harlan Indianapolis breeding colony, Fredrick, MD, USA) revealed a similar incidence, but a reduced duration of symptoms.

2.3.4.1 Hindpaw mechano-allodynia

Mechano-allodynia of the hindpaw was assessed by measuring the hindpaw withdrawal response to von Frey filament stimulation according to a modification of the method described by Chaplan et al. (1994). In brief, animals were placed in a Plexiglas® box (21×16×27 cm^3) with a wire grid bottom through which the von Frey filaments (Stoelting) were applied to the plantar surface of the hindpaw. Filaments were applied in either ascending or descending strength as necessary to determine the filament closest to the threshold of response. Each filament was applied 5 times; a response to three of the five applications was counted as positive. The minimum stimulus intensity was 0.25 g and the maximum was 15 g. Based on the response pattern and the force of the final filament, the 50% response threshold (grams) was calculated. The resulting pattern of positive and negative responses was tabulated using the convention, x=withdrawal, o=no withdrawal, and the 50% response threshold was interpolated

using the formula: 50% g threshold = $(10^{[xf + k\delta]})/ 10{,}000$, where x_f=value (in log units) of the final von Frey hair used; k=tabular value (see Chaplan et al., 1994) for pattern of positive/negative responses; and δ=mean difference (in log units) between stimuli (here 0.224). Hairs (nylon monofilaments; Stoelting, Woodale, IL, USA) were from the standard Semmes–Weinstein series (Semmes et al., 1960).

2.3.4.2 Hindpaw mechano-hyperalgesia

Mechano-hyperalgesia of the hindpaw was assessed using a modification of the pin prick method described by Tal & Bennett (1994). With the rats standing on the wire mesh floor and confined beneath an inverted plastic box (described above), the point of a blunted 23 gauge needle was applied to the skin of the heel (touching, but not penetrating). Normal rats respond with a very small and brief withdrawal. CPIP rats, like neuropathic rats (e.g. CCI rats), respond most often with a withdrawal that is clearly exaggerated in amplitude and duration. Behavioral responses to the pin prick were rated according to the following scale: 0=no response; 1=rapid paw flicking, stamping, or shaking (less than 1 s); 2=repeated paw stamping, shaking, or paw lift less than 3 s; 3=above behaviors or hindpaw licking for more than 3 s; 4=above behaviors for more than 3 s and hindpaw licking for more than 3 s. An additional point was added if any vocalizations occurred.

2.3.4.3 Hindpaw cold-allodynia

Cold-allodynia of the hindpaw was assessed using a modification of the acetone drop method described by Choi et al. (1994). With the rats standing on the wire mesh floor and confined beneath an inverted plastic cage, a drop of acetone was placed on the skin of the heel. Normal rats either ignore the stimulus or occasionally respond with a very small and brief withdrawal. CPIP rats, like CCI rats, respond most often with a withdrawal that is clearly exaggerated in amplitude and duration. Behavioral responses to the acetone drop were rated according to the same scale described above for the pin prick test.

2.3.4.4 Hindpaw heat-hyperalgesia

Heat-hyperalgesia of the hindpaw was tested using methods described by Hargreaves et al. (1988). Briefly, the rat was placed within a plastic compartment atop a glass floor; a light source beneath the floor was aimed at the skin of the fat part of the heel. The nocifensive withdrawal reflex interrupts the light reflected from the heel onto a photocell and automatically turns off the light and a timer. The intensity of the light was adjusted at the start of the experiment such that average baseline latencies were about 10 s and a cut-off latency of 20 s was imposed. Latency to withdrawal defines the heat–pain threshold. A latency score was based on the average of the two most consistent of three replicate recordings, which were obtained alternately from each hindpaw 5-min apart. Data were converted to percentage change from baseline since there was an approximately 1.75 s difference in the baseline latencies between the CPIP and sham rats for both ipsilateral and contralateral heat-hyperalgesia trials.

2.3.5 Histology

The tibial nerves of six animals were examined: four at 48 h and two at 7 days after IR. When deeply anesthetized following a sodium pentobarbital overdose (100 mg/kg, i.p.), the rats were perfused transcardially with 150 ml of a phosphate buffered solution containing 0.1% sodium nitrate, followed by 200 ml of freshly prepared 1% paraformaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer. Segments of the tibial nerve from the ankle (just proximal to the location, where the tourniquet had been applied) were harvested bilaterally and post-fixed in the same solution. Following incubation in 10% sucrose, the nerves were embedded in epoxy (Epon), sectioned at 1 μm, and stained with toluidine blue.

2.3.6 Free radical scavenger trial

Rats were administrated the agents TEMPOL or NAC to establish the potential anti-allodynic effects of free radical scavengers. TEMPOL is a nitroxide free radical scavenger (Thiemermann, 2003); NAC is a precursor of glutathione, an endogenous anti-oxidant (Skrzydlewska & Farbiszewski, 1999). After a baseline von Frey trial, rats received a 3 h tourniquet (O-ring) exposure and

reperfusion as described above. Rats were then tested for von Frey thresholds at 48 h after reperfusion, both before and 30 min after treatment with either TEMPOL (250 mg/kg, i.p.) or NAC (500 mg/kg, i.p.) doses that produce effective anti-oxidant effects *in vivo* (Cuzzocrea et al., 2001; Sener et al., 2003).

2.4 Results

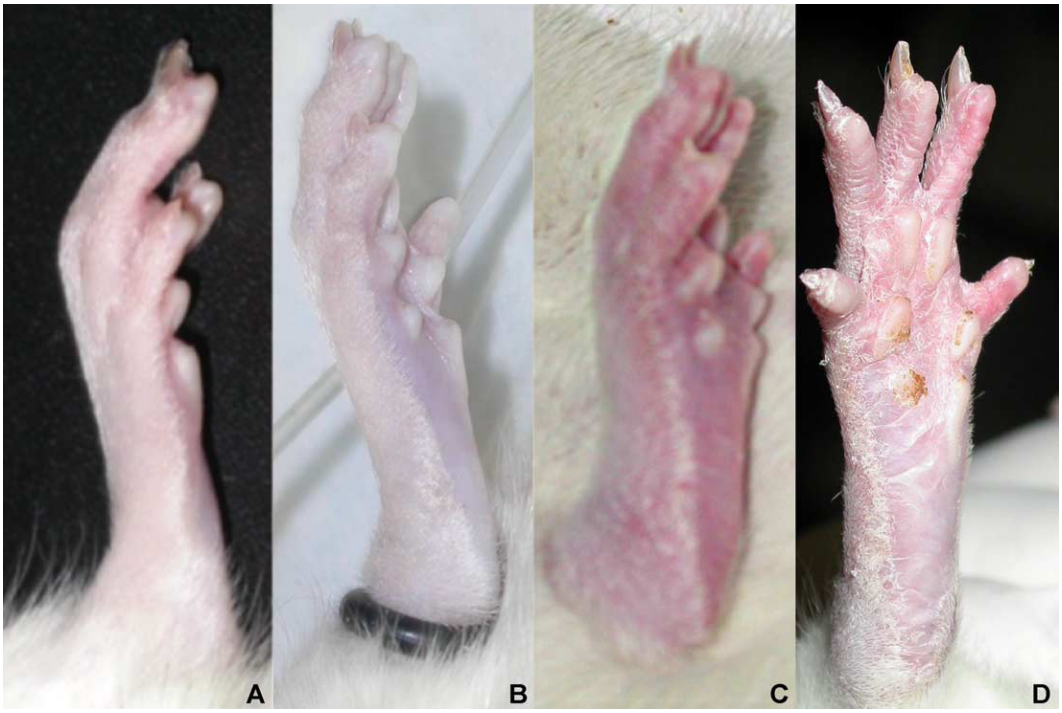

Figure 1. Representative photographs of rat hindpaws taken before tourniquet exposure (**A**), during tourniquet exposure (**B**), 5 min following reperfusion (**C**), and 24 h following perfusion (**D**). During tourniquet exposure (**B**), the hindpaw is cold and cyanotic, reflecting tissue hypoxia. Shortly after reperfusion (**C**) the hindpaw is hot, engorged with blood and oedematous, reflecting an intense reactive hyperemia. At 24 h post-reperfusion (**D**), the hyperemia and oedema subside, and the hindpaw appears dry and shiny.

2.4.1 Hyperaemia and plasma extravasation

Hindpaw temperature recordings were taken between 5 min and 4 h after reperfusion, and measurements were expressed as temperature difference between the ipsilateral and contralateral hindpaws for each rat. Two way repeated analysis of variance (ANOVA) revealed significant main effects of treatment group ($F_{1, 11}$= 6.06, $P < 0.05$) and time ($F_{4, 40} = 5.05$, $P < 0.01$), as well as a significant group×time interaction ($F_{4,40} = 7.14$, $P < 0.001$). Compared to sham rats for which hindpaw temperature did not vary significantly over the 4 h of testing, the mean temperature difference between the ipsilateral and contralateral hindpaws of CPIP rats was significantly elevated above baseline between 5 min and 2 h after reperfusion ($P < 0.05$, Dunnett's). The temperature difference peaked at 5 min following reperfusion, and returned to baseline levels by 4 h after reperfusion (Fig. 2A). In patients, a side-to-side temperature difference of 1 °C or greater is considered abnormal (Uematsu et al., 1988).

Hindpaw plasma extravasation assessments of CPIP rats were taken between 2, 12 and 24 h after reperfusion, and measurements were expressed as the difference in amount of extravasated blue dye between the ipsilateral and contralateral hindpaws for each rat. Compared to sham rats, which did not exhibit a significant difference between the ipsilateral and contralateral hindpaws at the 2 h time point, the mean difference in extravasated Evans Blue dye between the ipsilateral and contralateral hindpaws of CPIP rats was significantly elevated at 2 h after reperfusion ($F_{3,38} = 4.29$, $P < 0.05$), and returned to normal by 12 h after reperfusion (Fig. 2B).

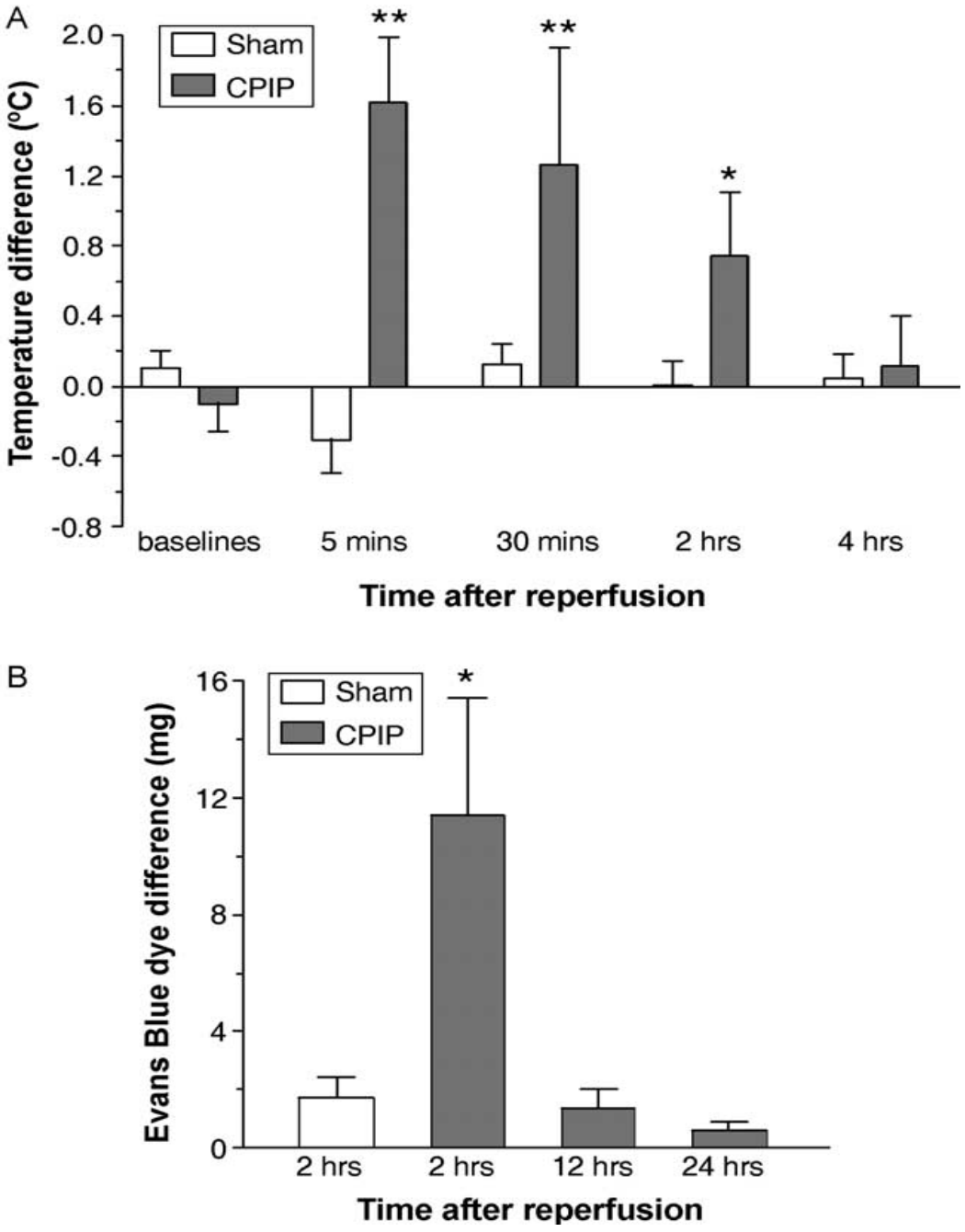

Figure 2. Time course of the hyperaemia and oedema in CPIP and sham rats, as measured as the temperature difference (**A**) and the difference in extravasated Evans Blue dye (**B**) between the ipsilateral and the contralateral hindpaws of CPIP and sham rats. A significant increase in the ipsilateral–contralateral hindpaw temperature difference for CPIP rats, but not shams, was observed between 5 min and 2 h after reperfusion (**A**). Compared to the measurement for shams at 2 h, there was also a significant increase in the difference of extravasated Evans Blue dye (ipsilateral–contralateral) in CPIP rats at 2 h (**B**) (*$P < 0.05$, **$P < 0.01$).

2.4.2 Mechanical and thermal sensitivity

2.4.2.1 Hindpaw mechano-allodynia

von Frey thresholds for both the ipsilateral (χ^2_7 = 1.04, P > 0.05) and contralateral (χ^2_7 = 0.78, P > 0.05; Friedman ANOVA) hindpaws of sham rats did not vary across the 4 weeks of testing. In contrast, CPIP rats developed mechano-allodynia over a prolonged period in both the ipsilateral (χ^2_7 = 31.6, P < 0.001) and contralateral (χ^2_7 = 17.0, P < 0.05) hindpaw, with more pronounced effects on the ipsilateral side. Ipsilateral mechano-allodynia was present within 8 h following reperfusion, peaked at 4 days, and persisted for at least 4 weeks after reperfusion (Fig. 3A). Contralateral mechano-allodynia was also present within 8 h following reperfusion, peaked at 2 days, and persisted for 2 weeks after reperfusion (Fig. 3B).

2.4.2.2 Hindpaw mechano-hyperalgesia

Nociceptive responses to pin prick for both the ipsilateral (χ^2_7 = 4.49, P > 0.05) and contralateral (χ^2_7 = 5.59, P > 0.05) hindpaws of sham rats did not vary across the 4 weeks of testing. In contrast, CPIP rats developed mechano-hyperalgesia over a prolonged period in both the ipsilateral (χ^2_7 = 28.0, P < 0.001) and contralateral (χ^2_7 = 21.7, P < 0.01) hindpaw, with more pronounced effects on the ipsilateral side. Ipsilateral mechano-hyperalgesia peaked at 8 h, and persisted for at least 4 weeks after reperfusion (Fig. 4A). Contralateral mechano-hyperalgesia was more sporadic, but was evident at 8 h, 4 days, and 2 and 4 weeks after reperfusion (Fig. 4B).

2.4.2.3 Hindpaw cold-allodynia

Responses to cold for both the ipsilateral (χ^2_7 = 1.26, P > 0.05) and contralateral (χ^2_7 = 2.50, P > 0.05) hindpaws of sham rats did not vary across the 4 weeks of testing. In contrast, CPIP rats developed cold-allodynia over a prolonged period in both the ipsilateral (χ^2_7 = 16.9, P < 0.05) and contralateral (χ^2_7 = 16.9, P < 0.05) hindpaw, with more pronounced effects on the ipsilateral side. Ipsilateral cold-allodynia was evident at 8 h, peaked at 2 weeks, and persisted for at least 4 weeks after reperfusion (Fig. 5A). Contralateral cold-allodynia was again more

sporadic, but was evident at 8 h, 4 days, and 2 and 4 weeks after reperfusion (Fig. 5B).

2.4.2.4 Hindpaw heat-hyperalgesia

The percentage change in nociceptive withdrawal latencies to noxious heat for both the ipsilateral and contralateral hindpaws of both sham and CPIP rats did not vary significantly across the 4 weeks of testing. Thus, two-way repeated ANOVA revealed non-significant main effects of treatment group ($F_{1,23} = 0.23$, $P > 0.05$) and time ($F_{6,138} = 0.69$, $P > 0.05$), as well as a non-significant interaction of group×time ($F_{6,138} = 0.48$, $P > 0.05$) for the ipsilateral latencies, and a non-significant main effects of group ($F_{1,23} = 1.02$, $P > 0.05$) and time ($F_{6,138} = 0.82$, $P > 0.05$), as well as a non-significant interaction of group×time ($F_{6,288} = 0.49$, $P > 0.05$) for the contralateral latencies. In general, there was a trend for the withdrawal latencies of CPIP animals to *increase* (data not shown), although this trend did not reach statistical significance.

2.4.3 Histology

Light-microscopic examination found no evidence of degeneration in the ipsilateral tibial nerves of three of the four rats examined 48 h after IR, in either of the rats examined at 7 days after IR, or in any nerves taken from the contralateral side. The ipsilateral tibial nerve of one rat examined at 48 h after IR had about a dozen myelinated axonal profiles, scattered throughout the endoneurial compartment, that did not have a clearly delineated central core of axoplasm; we are uncertain as to whether these fibers were degenerating or whether their appearance was due to fixation or staining artifact.

2.4.4 Free radical scavenger trial

As demonstrated in the time course trials, 48 h following hindpaw IR both ipsilateral and contralateral von Frey thresholds were significantly lower than baseline (Fig. 6), indicative of mechano-allodynia in CPIP rats. Conversely, 30 min following treatment with either NAC (Fig. 6A) or TEMPOL (Fig. 6B), both ipsilateral and contralateral von Frey thresholds of CPIP rats were not different from baseline, suggesting that both NAC and TEMPOL reversed the mechano-allodynia in CPIP rats.

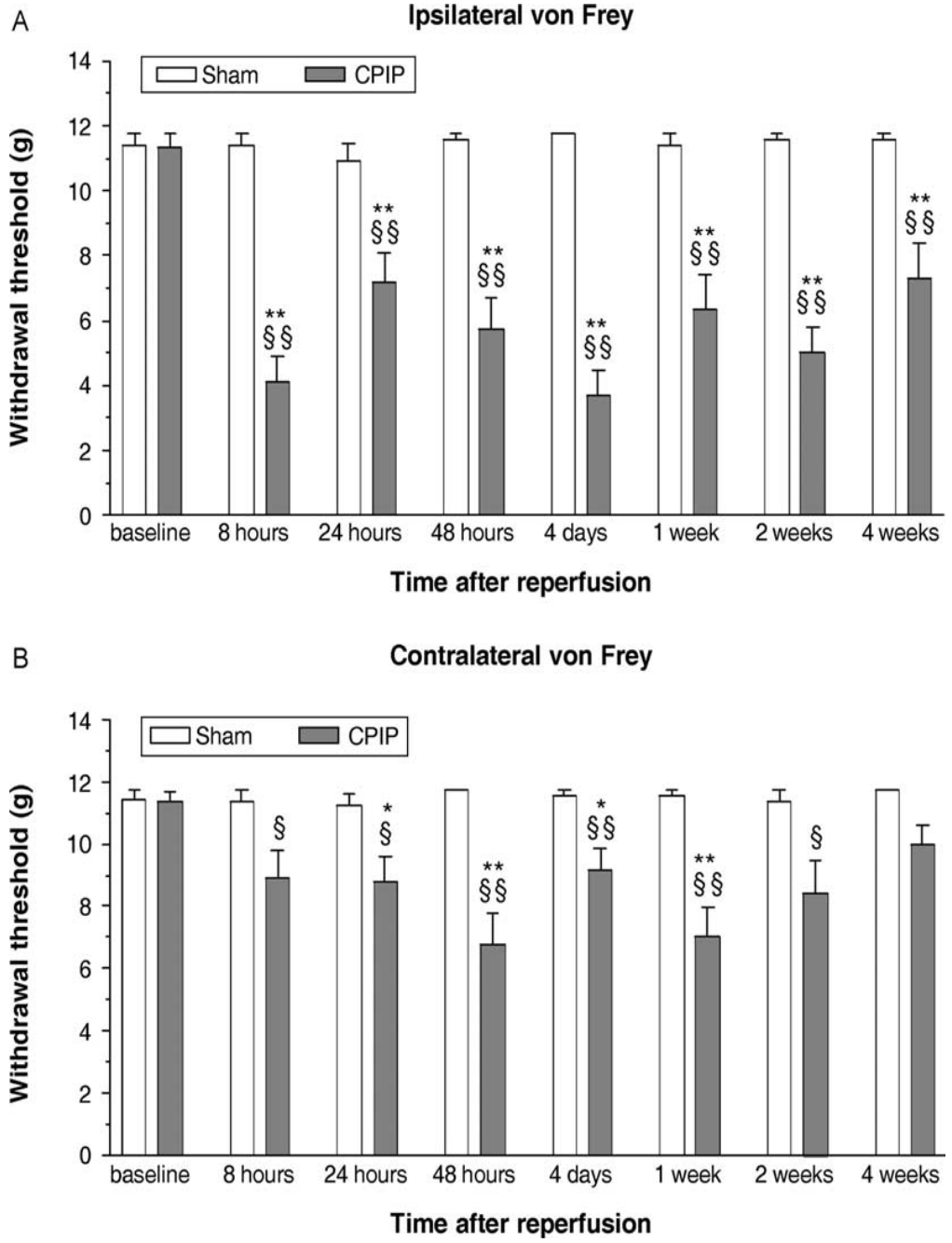

Figure 3. Time course of mechano-allodynia in the ipsilateral (A) and contralateral (B) hindpaws of CPIP and sham rats, as determined by the von Frey test. Ipsilateral and contralateral withdrawal thresholds of sham rats did not change throughout the 4 weeks of testing. Withdrawal thresholds of CPIP rats were significantly reduced between 8 h and 4 weeks after reperfusion, ipsilaterally, and between 8 h and 2 weeks after reperfusion, contralaterally (*$P <$ 0.05, **$P < 0.01$ from sham; $^{\S}P < 0.05$, $^{\S\S}P < 0.01$ from baseline).

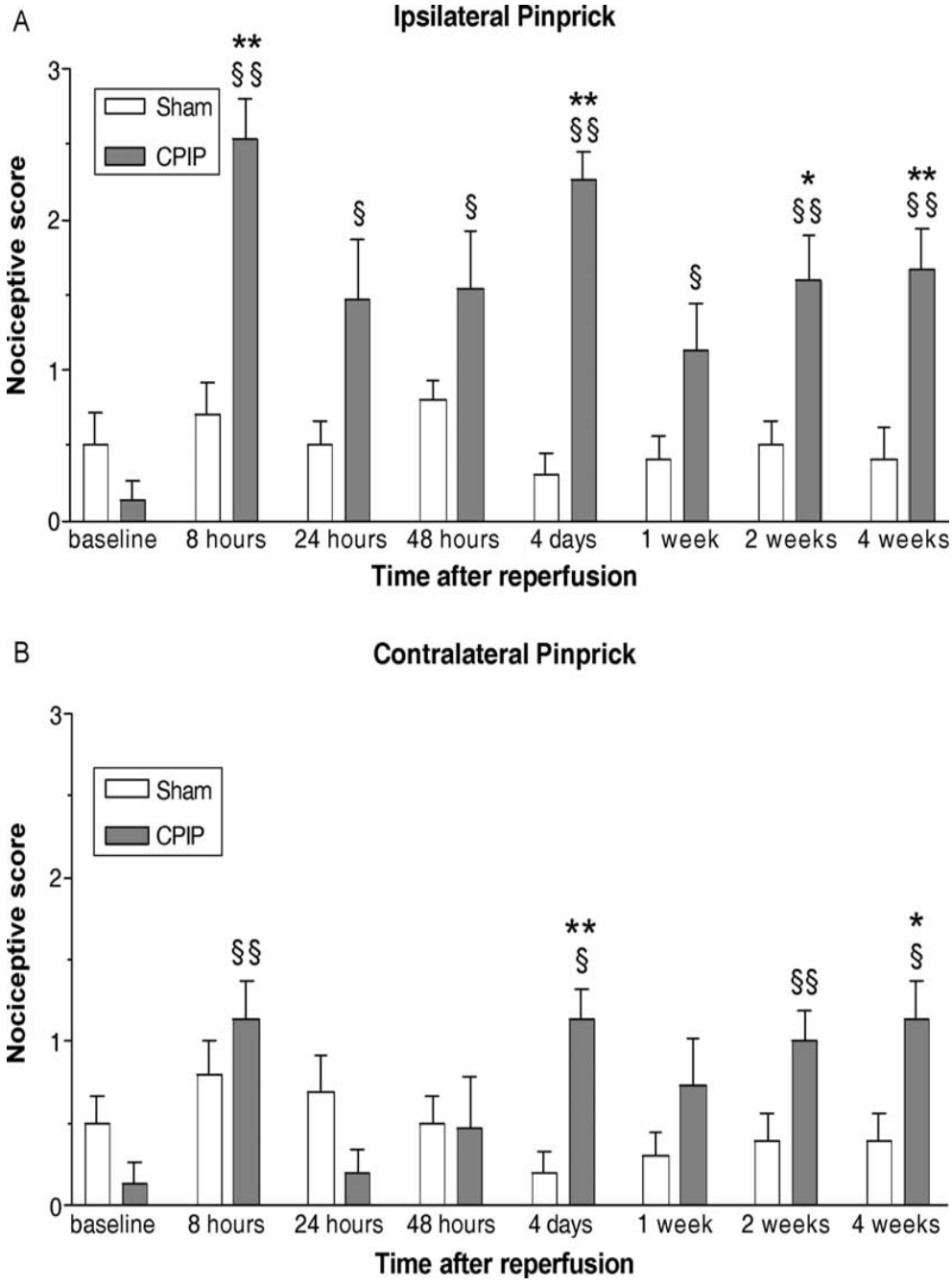

Figure 4. Time course of mechano-hyperalgesia in the ipsilateral (**A**) and contralateral (**B**) hindpaws of CPIP and sham rats, as determined by the pin prick test. Ipsilateral and contralateral nociceptive scores of sham rats did not change throughout the 4 weeks of testing. Nociceptive scores of CPIP rats were significantly elevated between 8 h and 4 weeks after reperfusion, ipsilaterally, and at 8 h, 4 days and 2–4 weeks after reperfusion, contralaterally (*$P < 0.05$, **$P < 0.01$ from sham; $^{\S}P < 0.05$, $^{\S\S}P < 0.01$ from baseline).

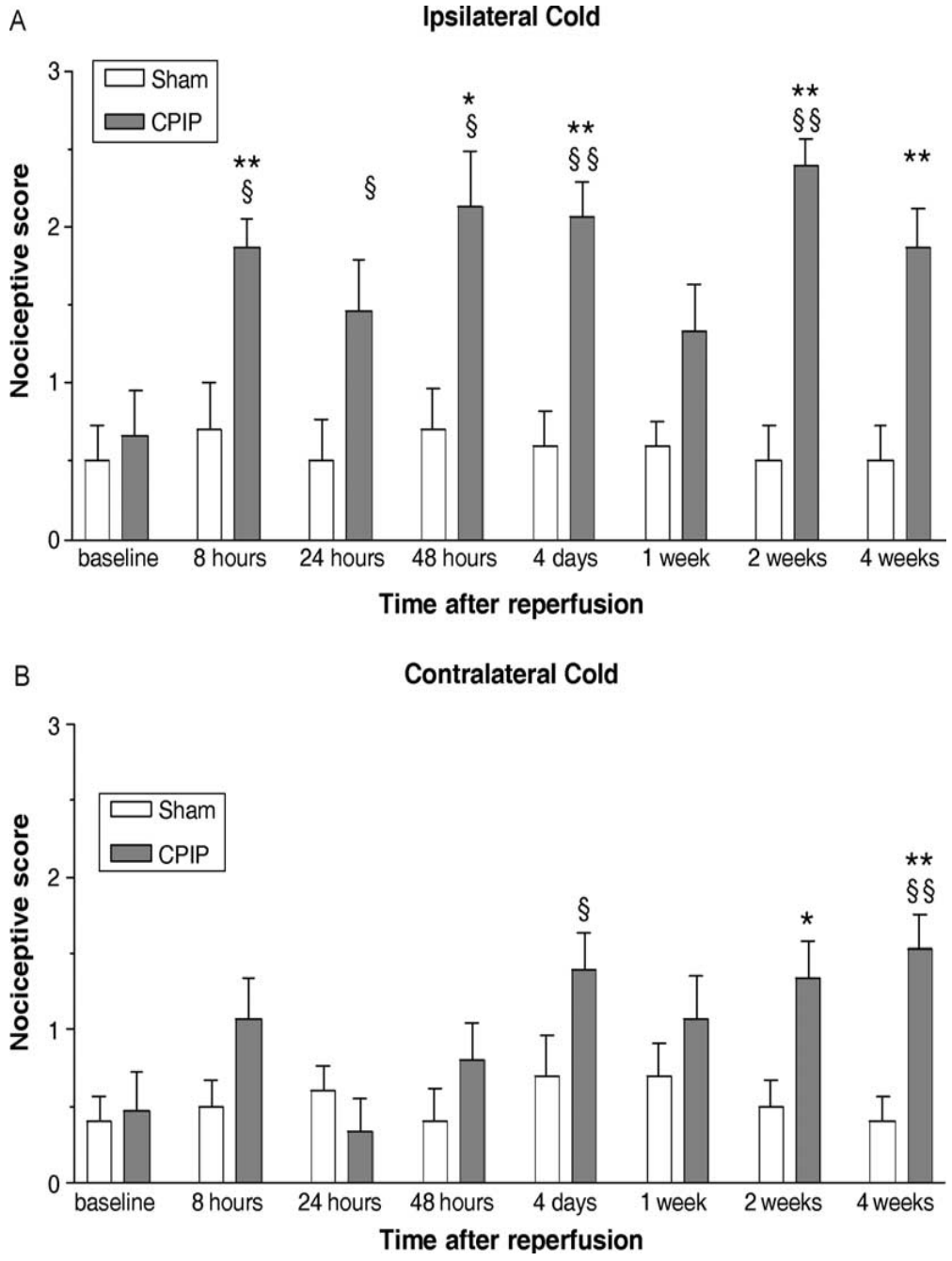

Figure 5. Time course of cold-allodynia in the ipsilateral (**A**) and contralateral (**B**) hindpaws of CPIP and sham rats, as determined by the acetone drop test. Ipsilateral and contralateral nociceptive scores of sham rats did not change throughout the 4 weeks of testing. Nociceptive scores of CPIP rats were significantly elevated between 8 h and 4 days, and 2–4 weeks after reperfusion, ipsilaterally, and at 4 days and 2–4 weeks after reperfusion, contralaterally (*$P <$ 0.05, **$P < 0.01$ from sham; $^{\S}P < 0.05$, $^{\S\S}P < 0.01$ from baseline).

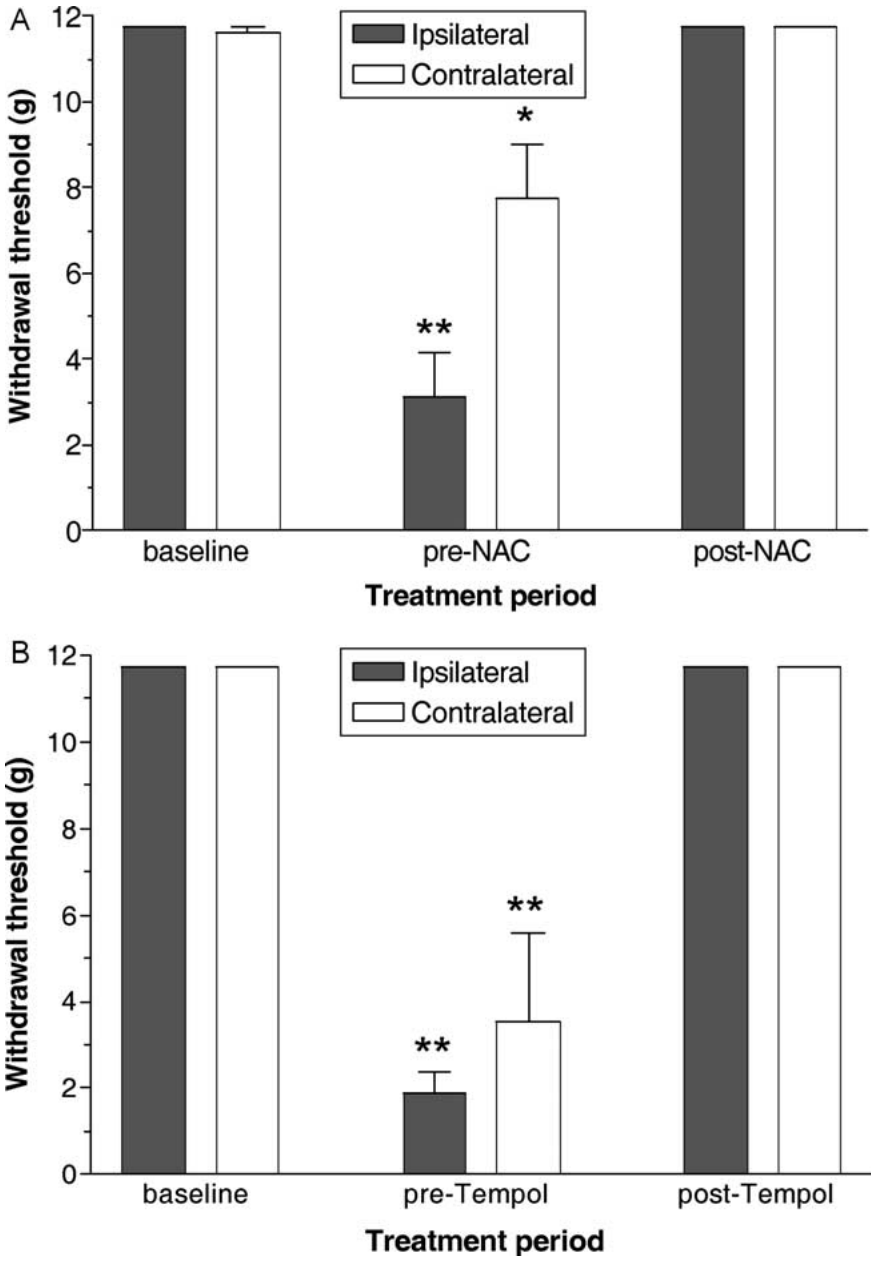

Figure 6. Comparison of the mechanico-allodynia observed before and after post-treatment with NAC (**A**) or TEMPOL (**B**) 48 h after reperfusion in CPIP rats. Both NAC (500 mg/kg, i.p.) and TEMPOL (250 mg/kg, i.p.) reversed mechano-allodynia in the ipsilateral and contralateral hindpaws (*$P < 0.05$, **$P < 0.01$).

2.5 Discussion

Rats exposed to prolonged hindpaw IR exhibited hyperemia and plasma extravasation in the ischemic hindpaw, acutely, and neuropathic pain-like symptoms, including hyperalgesia to noxious mechanical stimulation, cold-allodynia and mechano-allodynia, but not heat-hyperalgesia, in both the ischemic, and to a lesser extent, the contralateral hindpaw, chronically. However, there were no indications of nerve injury in CPIP rats. There was no loss or abnormality of motor function, no evidence of sensory anesthesia, and no microscopic evidence of nerve injury in the majority of the cases examined. Determination of the status of the unmyelinated C-fiber axons will require an electron microscopic study.

The development of CPIP as an animal model of CRPS-I is a physical injury model for this syndrome in which nerve injury is not evident. Other animal models of CRPS-I have been developed including that produced by sustained (10 min) tetanic stimulation of the sciatic nerve in the rat (Vatine et al., 1998; Vatine et al., 2001), and that induced by continuous hindlimb intra-arterial infusion of a free radical donor, tert-butylhydroperoxide (tert-BuOOH) (van der Laan et al., 1997a; van der Laan et al., 1997b; van der Laan et al., 1998a). An interesting parallel between the CPIP and the free radical donor model is the very significant appearance of bilateral symptoms. van der Laan et al. (1997b) found significant mechano-allodynia in the hindpaw contralateral to the tert-BuOOH infusion between 7 and 28 days after the infusion. Although contralateral effects have been described sporadically following various models of neuropathic and inflammatory pain (see Koltzenburg et al., 1999 for review), rarely are the effects so robust and long-lasting, as demonstrated in these two models. The robustness of these bilateral effects parallel findings of CRPS-I spreading to the contralateral side of some patients, reportedly as high as 16% (Allen et al., 1999; Maleki et al., 2000). We expect that the robust contralateral effects may depend on the higher degree of central sensitization that is obtained after injury of muscle tissue, as opposed to cutaneous tissue (Woolf & Wall, 1986).

The strength of the present CPIP model is that CRPS-I-like symptoms are induced by a physical injury that is comparable to those injuries seen in CRPS-I

patients. CRPS-I commonly follows fractures, sprains, contusions and crush injuries, arthroscopic surgery, overly tight casting, and other oedematous soft tissue injuries (Allen et al., 1999; Galer et al., 2000; Sandroni et al., 2003). A common feature of all these conditions is an early inflammatory response that has the potential to produce microvascular and ischemic changes in various tissues. Interestingly, the incidence of vascular complications after arthroscopic surgery (including RSD) are dramatically increased when a tourniquet time of 60 min or longer is used (Sherman et al., 1986). CRPS-I is not common after tourniquet application, chiefly because surgeons are aware of the dangers of prolonged tourniquet exposure. Typically tourniquets are not used longer than 2 h, and blood flow is intermittently re-established for the longer tourniquet times (Fletcher and Healy, 1983). Despite this, it has been established that after arthroscopic surgery there is typically more pain (Berga et al., 2002) and more dysfunction (Gutin et al., 1991) following tourniquet use.

We suggest that the symptomology that occurs after CPIP (early hyperemia and oedema followed by long-lasting mechanical and cold-allodynia/hyperalgesia) resembles the two prominent phases of CRPS-I in humans (Birklein et al., 2000a; Birklein et al., 2000b; Wasner et al., 2001a; Wasner et al., 2001b). However, in patients hyperemia and oedema are not always present, and may be brief, or alternatively may be prolonged for many months or even years. There are even those who argue that CRPS-I limbs do not always progress from a hot oedematous stage to a cold "vasoconstrictive" stage (Bruehl et al., 2002). Nonetheless, the fact that IR injury leads to a persistent pain syndrome in rats, brings to light the possibility that similar mechanisms may contribute to some symptoms in CPIP rats and CRPS-I patients.

We expect that the extensive, but brief, ischemia in CPIP rats produces only an acute inflammatory response, while less extensive, but more prolonged, ischemia may result in a longer lasting inflammatory period in CRPS-I patients. Although the extent and course of the process may vary, the underlying mechanisms may be quite similar. The fact that CPIP rats do not exhibit a later cold limb or motor disturbances may reflect the relatively short period for which

the animals were observed. However, prolonged or extensive tissue ischemia and reperfusion can produce an increasing myofascial dysfunction leading to motor disturbances (Hnik et al., 1997; Labbe et al., 1987). Hyperhidrosis that occurs in some CRPS-I patients does not appear to be evident in CPIP rats. However, rats have very few sweat glands, and they do not use sweating for temperature regulation. Thus, one would not expect to see sweating in association with vasodilatation, as occurs in humans (Jänig & Häbler, 2003). It is also true that as high as 50% of CRPS-I patients exhibit heat-hyperalgesia (Price et al., 1992; Tahmoush et al., 2000), while CPIP rats do not. Clearly, IR injury may not explain all symptoms experienced by all CRPS-I patients, which are arguably a fairly heterogeneous group. This heterogeneity is highlighted by other reports indicating that like for CPIP rats, heat-hyperalgesia is absent or infrequent in CRPS-I patients (see Guo et al., 2004).

The results here suggest we may be able to learn more about CRPS-I by studying the inflammatory processes that follow IR. Hindlimb IR produces various effects depending on the area and length of ischemia. When blood flow is occluded to the entire hindlimb, there is damage to muscle after 4 h and to peripheral nerves after 8 h of IR (Labbe et al., 1987; Steinau et al., 1988). Between 2 and 4 h of IR, there are microcirculatory changes (thrombosis, capillary endothelial cell swelling, leukocyte plugging) that result in increased vascular permeability to plasma proteins, interstitial oedema (Harris et al., 1997 and Strock and Manjo, 1969), and arteriovenous shunting (Kennedy et al., 1981). Before 4 h there is minor damage to muscle, but after 4 h the loss of nutrient blood flow causes muscle necrosis (Makitie & Teravainen, 1977). Ischemic conditions secondary to oedema accumulation may lead to a compartment syndrome, where increased tissue pressure in an anatomical compartment compromises blood flow to muscles, nerves and bone causing tissue damage (Perry, 1988). Untreated compartment syndrome in muscle will lead to ischemic contracture—causing muscle stiffness and deformity (Hoover & Siefert, 2000). More extensive and/or longer ischemic periods lead to oxidative stress (lipid

peroxidation) that damages the blood nerve barrier and causes endoneurial oedema and nerve fiber degeneration (Saray et al., 1999).

We propose that CRPS-I, in some cases, depends on a microcirculatory abnormality that occurs following IR and persistent inflammation that occur after the initial insult. It may be significant that CRPS-I often follows fractures, sprains and arthroscopic surgery of knees and elbows (Allen et al., 1999; Galer et al., 2000). One can imagine that such injuries could be especially likely to produce compartment-like syndromes, where oedema accumulates within muscle, joint capsules or other anatomical compartments, and leads to significant tissue ischemia. These events may lead to a persistent state of tissue ischemia, or borderline ischemia, which is likely to sensitize and activate the afferent innervation of the tissue. If true for muscle and/or periosteal nociceptors, this would cause a persistent, deep pain sensation. Activation of muscle or periosteal nociceptors, or skin C-fibers, may also lead to a central sensitization that would contribute to mechanical allodynia/hyperalgesia and cold-allodynia. Importantly, conditioning stimulation of muscle C-fibers produces greater central sensitization than does stimulation of cutaneous C-fibers (Wall & Woolf, 1984). Of note, strictly cutaneous injuries (e.g. burns and lacerations) are very infrequently cited as antecedents of CRPS-I.

Microcirculatory abnormalities may maintain CRPS-I symptoms. CRPS-I patients commonly report that exercise worsens their pain (Oyen et al., 1993), as would be expected if their muscles were borderline ischemic. Ischemia in bone and periosteal tissues might underlie the osteoporosis often found in CRPS-I patients (Kozin et al., 1976a; Kozin et al., 1976b; Mailis et al., 1994; Sudeck, 1902). Activity in sympathetic fibers of the affected limb would exacerbate any underlying ischemic condition, and this would explain the often important, but not necessarily causative, contribution of the SNS to CRPS-I (Baron and Maier, 1996; Blumberg et al., 1997; Bonica, 1979; Wasner et al., 2001a; Wasner et al., 2001b).

Prolonged hindlimb IR has been shown to produce a well-documented cascade of inflammatory events, with a key role for reactive oxygen species (Blaisdell, 2002; Yassin, 1997). Hindlimb IR results in the production of the

oxidants, superoxide, hydrogen peroxide, hydroxyl radical, perhydroxyl radical, singlet oxygen and peroxynitrite anion, initiated by the enzymes XO (Kellog, 1975; McCord, 1987) or NADPH oxidase (Inauen et al., 1989; Partrick et al., 1996). Xanthine oxidase and lipid peroxidase activity is increased in sciatic nerve of CCI rats (Khalil & Khodr, 2001; Khalil et al., 1999). Our investigations demonstrating that CPIP symptoms are reduced by post-treatment with free radical scavengers stresses the role of oxidants in the maintenance of neuropathic pain-like symptoms in this model of CRPS-I as well.

2.6 Acknowledgements

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Intervening Section 1:

While there have been many well-characterized and widely used animal models of CRPS-II and neuropathic pain, very few animal models have been developed for CRPS-I, and none of these have been well characterized. Therefore, the overall aim in chapter 2 was to develop an animal model of CRPS-I by a novel method, tourniquet I-R injury. The three-hour ischemia with an O-ring induced clear mechanical allodynia, mechanical hyperalgesia, and cold allodynia in 70% of Long-Evans rats that persisted for at least 4 weeks. Short-lasting hyperemia and oedema was also present, as well as no signs of major nerve injury underneath the tourniquet. Consistent with the prominent role of free radicals in I-R injury, a small trial with 2 free radical scavengers was performed and shown to successfully reverse mechanical allodynia. Hence, we propose that 3-hour tourniquet ischemia induces similiarities to clinical signs and symptoms of CRPS-I, and that it can be considered an animal model of CRPS that we named chronic post-ischemia pain (CPIP).

While historically CRPS-I was thought to involve sympathetic dysfunction and respond to anti-sympathetic treatments, it is animal models of CRPS-II and neuropathic pain induced by peripheral nerve injury that have often found that the SNS contributes to the ongoing pain. Sympathetic sprouting, changes in adrenergic receptor distribution in spinal cord and DRG, and hypersensitivity of afferent nerves to adrenergic agents have been found and suggested to contribute to abnormal sympathetic-sensory coupling. Surgical and chemical sympathectomies have also been shown to reduce mechanical hypersensitivity, cold allodynia, and heat hyperalgesia in some of these animal models. α-adrenergic antagonists have also been found to reduce neuropathic pain. This has often been suggested to resemble patients that respond to anti-sympathetic procedures, and classified as having SMP.

Interestingly, the few animal models of CRPS-I that have been developed have not examined whether anti-sympathetic procedures provide anti-nociception. Hence, to further characterize CPIP, the next aim was test whether CPIP rats

display SMP. Regional sympathetic blockade with guanethidine, systemic phentolamine, and other α-adrenergic antagonists are often used in CRPS patients with SMP. Hence, we examined these agents for their anti-allodynic effects in CPIP rats at 2 days and 7 days post-reperfusion. We hypothesized that the regional vasodilatory effect of these anti-sympathetic treatments, which is seen after a successful anti-sympathetic procedure clinically, may be a particularly relevant mechanism of anti-nociception after I-R injury. To further test this hypothesis, we also examined whether vasodilatation produced by an NO donor would also alleviate mechanical allodynia in CPIP rats.

Chapter 3

Sympathetic vasoconstrictor antagonism and vasodilatation relieve mechanical allodynia in rats with chronic post-ischemia pain

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3.1 Abstract

Chronic pain that responds to antisympathetic treatments and α-adrenergic antagonists is clinically referred to as sympathetically maintained pain. Animal models of neuropathic pain have shown mixed results in terms of antinociceptive effectiveness of antisympathetic agents. The effectiveness of these agents have not been yet investigated in animal models of CRPS-I. In this study, we examined the effectiveness of antisympathetic agents and sympathetic vasoconstrictor antagonists, as well as agents that are vasodilators, in relieving mechanical allodynia in a recently developed animal model of CRPS-I (chronic post-ischemia pain or CPIP) produced by 3 hours of hind paw I-R injury. Systemic guanethidine, phentolamine, clonidine, and prazosin are effective in reducing mechanical allodynia particularly at 2 days after reperfusion, and less so at 7 days after reperfusion. A NO donor vasodilator, SIN-1, also reduces mechanical allodynia more effectively at 2 days after reperfusion, but not at 7 days after reperfusion. These results suggest that the pain of CPIP, and possibly also CRPS-I, is relieved by reducing sympathetically mediated vasoconstriction, or enhancing vasodilatation.

Perspective: The results of this study indicate that sympathetic block, or administration of α_1-adrenergic antagonists, clonidine, or a NO donor, relieve allodynia in an animal model of CRPS-I. Thus, the pain of CRPS-I may depend on enhanced vasoconstrictor responsiveness, which may be relieved by blocking sympathetic efferent-dependent vasoconstriction, or by enhancing NO–dependent vasodilatation.

Key words: CRPS, ischemia-reperfusion injury, neuropathic pain, allodynia, sympathetic block, adrenergic receptors.

3.2 Introduction

CRPS-I is a disorder that occurs after fracture, soft tissue or crush injury (De Mos et al., 2007). Symptoms of CRPS-I include spontaneous burning (cutaneous) and aching (deep) pain, hyperalgesia, allodynia, and disorders of vasomotor and sudomotor regulation (Jänig & Baron, 2003; Rowbotham, 2006). CRPS-II is similar but also exhibits a clinically verified nerve injury (Wasner et al., 1998). Although controversial, sympathetic blocks are often reported to relieve CRPS pain (Cepeda et al., 2002; Kaplan et al., 1996; Stanton-Hicks, 2003). CRPS pain is also relieved with α-adrenergic antagonists such as phentolamine or phenoxybenzamine (Muizelaar et al., 1997; Raja et al., 1991), agents that have been used as diagnostic tools for identifying so-called SMP (Arnér, 1991; Wehnert et al., 2002). Although clinical studies have not determined the specific mechanisms of SMP, CRPS-I patients display abnormal responses to sympathetic stimulation (Wasner et al., 2001b) and enhanced vasoconstrictive responses to NE (Arnold et al., 1993; Ackerman et al., 2005), as well as painful responses to intradermal NE (Torebjörk et al., 1995). Pain relief in CRPS patients usually follows warming of the affected limb (Treede et al., 1992; Baron & Maier, 1996; Schürmann et al., 2001a), suggesting that vasodilatation may be important to pain relief by sympathetic blockade in CRPS (Treede et al., 1992). Sympathectomy also relieves neuropathic pain in animals with SNL, CCI, and PSNL (Shir & Seltzer, 1991; Kim et al., 1997, however, see Ringkamp et al, 1999a) but not cryoneurolysis or sural and tibial nerve transaction (Willenbring et al., 1995; Han et al., 2006). α-adrenergic antagonists also relieve neuropathic pain in animal models (Lee et al., 1999, Kim et al., 2005a; Hord et al., 2001, but see Park et al, 2000; Ringkamp et al, 1999b). Mechanisms that have been suggested for SMP in these models of CRPS-II include sympathetic efferent–primary afferent coupling (Chen et al., 1996), possibly after sympathetic fiber sprouting (Lee et al., 1998; Ramer & Bisby, 1997), or *de novo* adrenergic sensitivity in damaged afferents (Wall & Gutnick, 1974; Devor & Jänig, 1981), DRGs (Devor et al., 1994; Michaelis et al., 1996), or afferent nociceptors (Sato & Perl, 1991; O'Halloran & Perl, 1997). De novo adrenergic sensitivity may depend on

upregulation of adrenoceptors on primary afferent nerve fibers (Cho et al, 1997; Birder & Perl, 1999; Xie et al., 2001a) or other indirect mechanisms such as tissue ischemia due to enhanced vasoconstrictive responsiveness to NE. Importantly, rats with CCI have impaired vasoconstriction to sympathetic stimulation and enhanced vasoconstriction to exogenous NE (Kurvers et al., 1997; Kurvers et al., 1998). We recently introduced CPIP as an animal model of CRPS-I produced after hind paw I-R injury (Coderre et al., 2004). This animal model shows signs of I-R injury such as no-reflow and vascular abnormalities, as well as nociceptive and vascular hypersensitivity to NE (Laferrière et al., 2007; Xanthos et al., 2008). The pain-relieving effects of antisympathetic agents have not previously been tested in animal models of CRPS-I (Van der Laan et al., 1997a; Guo et al., 2006; Gradl et al., 2007). In this report, we examine whether antisympathetic drugs, and agents that are vasodilators, are effective in reducing painful symptoms in this model. Thus, we test the effectiveness of sympathetic block with guanethidine, or systemic treatments with the α_1- and α_2-adrenergic antagonists prazosin and yohimbine, the α_2-adrenergic agonist clonidine, and a NO donor, in relieving mechanical allodynia in CPIP rats at 2 and 7 days after reperfusion.

3.3 Materials and methods

3.3.1 Animals

Male Long-Evans hooded rats (275–325 g; Charles River, Québec, Canada) were housed in groups of 3 to 4, with food and water available ad libitum, on a 12:12-hour light:dark cycle. All treatments and testing procedures were approved by the Animal Care Committee at McGill University, and conformed to the ethical guidelines of the Canadian Council on Animal Care and the IASP.

3.3.2 Animal model of CRPS-I

Chronic postischemia pain was generated after exposure to prolonged hind paw ischemia and reperfusion as described in Coderre et al.14 Briefly, rats were anesthetized over a 3- to 4-hour period with a bolus (40 mg/kg, i.p.) and chronic i.p. infusion of sodium pentobarbital for 2 hours (13 mg/h for the first hour, 6.5 mg/h for the second hour). After induction of anesthesia, a Nitrile 70 Durometer

O-ring (O-Rings West, Seattle, WA, USA) with a 7/32-inch internal diameter was placed around the rat's left hind limb just proximal to the ankle joint for 3 hours. We standardized the position of the O-ring to a point on the limb just proximal to the medial malleolus of the tibia. The termination of sodium pentobarbital anesthesia was timed so that rats recovered fully within 30 to 60 minutes after reperfusion, which occurred immediately after removal of the O-ring. Sham rats received exactly the same treatment, except that the O-ring was cut, so that it only loosely surrounded the ankle and did not occlude blood flow to the hind paw.

3.3.3 Mechanical sensitivity nociceptive testing

Hind paw mechanical thresholds were assessed by measuring the withdrawal response to von Frey filament stimulation according to a modification of the up/down method described by Chaplan et al. (1994). In brief, rats were placed in a plexiglas box (21 x 16 x 27 cm^3) with a wire grid bottom through which the von Frey filaments (nylon monofilaments; Stoelting, Woodale, IL, USA) were applied to the plantar surface of the hind paw. Filaments were applied in either ascending or descending strength as necessary to determine the filament closest to the threshold of response. Each filament was applied once for 10 seconds to the center of the paw between the pads, and a lower intensity hair followed each positive response, whereas a higher intensity hair followed each negative response (until 5 responses were recorded after a first change in response). The minimum stimulus intensity was 0.25 g and the maximum was 15 g. Based on the response pattern and the force of the final filament, the 50% response threshold (grams) was calculated. The resulting pattern of positive and negative responses was tabulated, and the 50% response threshold was interpolated using the formula 50% g threshold = $(10^{[xf + k\delta]})/ 10{,}000$, where x_f = the value (in log units) of the final von Frey hair used; k = tabular value (Chaplan et al., 1994) for pattern of positive/ negative responses; and δ = mean difference (in log units) between stimuli (here, 0.224). Hairs were from the standard Semmes-Weinstein series (Semmes et al., 1960).

3.3.4 Drugs

Guanethidine, phentolamine, prazosin, yohimbine, and clonidine were all obtained from Sigma (Oakville, Ontario, Canada). SIN-1 was obtained from Tocris Bioscience (Ellisville, MO, USA). All agents were diluted in a 0.9% saline vehicle (VEH).

3.3.5 Drug administration protocol

Mechanical sensitivity was tested before CPIP induction and before drug administration on day 2 or day 7 days after reperfusion (predrug) and at various times after drug administration. To avoid testing rats that did not exhibit allodynia, it was decided a priori that, for all groups, only rats with postreperfusion (predrug) von Frey thresholds less than 6 g were used in the drug trials (accordingly, the mean and the standard error of the mean (SEM) for baseline von Frey thresholds only include rats that were subsequently given drug treatments). For sympathetic block experiments, VEH or guanethidine (30 mg/kg) was injected s.c. twice (separated by 24 hours) on either days 2 and 3 or days 7 and 8 after reperfusion. Mechanical allodynia was tested 4 hoursafter the second injection on day 3 (for days 2–3 guanethidine) or day 8 after reperfusion (for days 7–8 guanethidine). Separate groups of rats that received VEH or guanethidine injections on days 2 to 3 were tested on days 3, 5, 9, and 14 after reperfusion. Previous studies have shown that even a single dose of systemic guanethidine is sufficient for long-term sympathetic blockade (Maxwell et al., 1960; Kim et al., 1993). For dose-effect studies with phentolamine (1–5 mg/kg), prazosin (1–5 mg/kg), yohimbine (1–5 mg/kg), clonidine (0.01– 0.1 mg/kg), and SIN-1 (1–10 mg/kg), the drugs were administered i.p., and mechanical allodynia was tested 20 minutes later on either day 2 or 7 after reperfusion. Separate rats were tested on days 2 and 7. For time-course studies, rats were tested over a 3-hour period on day 2 after reperfusion with the highest dose of each drug (except guanethidine). All drug dosages were selected on the basis of previous studies examining the effects of these agents on nociception (Ringkamp et al., 1999b; Park et al., 2000; Shannon & Lutz, 2000; Hord et al., 2001; Sluka & Chandran, 2002; Martin & Persinger, 2004; Kim et al., 2005a). Furthermore, all drug doses that were used

were determined not to induce significant abnormalities in the rotorod test (see Appendix B). The highest doses of phentolamine, prazosin, and yohimbine used produced a significant inhibition of NE-induced reductions in hind paw blood flow as measured by laser Doppler flowmetry (data not shown). All experiments were performed by using a randomized blocks design, and at the time of testing the experimenter was blind to the animal's treatment. Six to 7 rats were used per group.

3.3.6 Statistics

Group comparisons were analyzed using a 2-way repeated-measures ANOVA followed by Fisher's least significant difference (LSD) post hoc tests. Preinjury baseline von Frey thresholds are included in the figures for each drug trial but are not included in the statistical analyses.

3.4 Results

Approximately 70% of rats subjected to the CPIP procedure displayed mechanical allodynia (von Frey threshold below 6 g) and were used in the drug trials. There were no significant differences between groups in baseline paw-withdrawal thresholds or predrug trial thresholds at 2 days or 7 days after reperfusion.

3.4.1 Effect of sympathetic block on CPIP mechanical allodynia

Fig 1A shows von Frey thresholds of CPIP rats before and after guanethidine or VEH treatment starting on day 2 after reperfusion. Two-way ANOVA reveals significant main effects of group ($F_{1, 23} = 5.77$, $P < .05$) and time ($F_{1, 23} = 4.85$, $P < .05$) and a significant group × time interaction ($F_{1, 23} = 19.33$, $P < .01$). The VEH injection did not produce a significant antiallodynic effect ($P > .05$); however, guanethidine-treated rats displayed a significant increase in paw withdrawal threshold (PWT) after the 2-day guanethidine treatment ($P < .01$). Continuing daily guanethidine treatment for up to 7 days did not result in any further reduction of mechanical allodynia (data not shown). Fig 1B shows von Frey thresholds of CPIP rats before and after guanethidine or VEH treatment starting on day 7 after reperfusion. Two-way ANOVA reveals nonsignificant main effects of group ($F_{1, 23} = 0.05$, $P > .05$) and time ($F_{1, 23} = 1.12$, $P > .05$) and a

nonsignificant group × time interaction ($F_{1, 23} = 0.12$, $P > .05$). This suggests that at this time point on day 8, the 2-day guanethidine treatment did not reduce mechanical allodynia. Fig 1C shows von Frey thresholds of CPIP rats before and for 14 days after VEH or guanethidine treatment on days 2 and 3 after reperfusion. Two-way ANOVA reveals a significant effect of group ($F_{1, 64} = 7.20$; $P < .05$) and time ($F_{4, 64} = 2.81$; $P < .05$), and a nonsignificant group × time interaction ($F_{4, 64} = 1.97$, $P > .05$). The guanethidine treatment resulted in significant increase of PWT as compared with day 2 predrug baseline, at day 3 ($P < .01$), day 5 ($P < .05$), and day 14 ($P < .01$). The VEH treatment did not result in any significant increase of PWT at any of the time points tested.

3.4.2 Effect of the mixed α_1-/α_2-adrenergic antagonist phentolamine on CPIP mechanical allodynia

Fig 2A shows the von Frey thresholds of 2-day CPIP rats before and 20 minutes after treatment with VEH or 1, 2.5, and 5 mg/kg phentolamine. Two-way ANOVA revealed a significant main effect of time (pre-post) ($F_{1, 47} = 8.05$, $P < .05$), a nonsignificant main effect of dose ($F_{3, 47} = 0.58$, $P > .05$), and a nonsignificant time × dose interaction ($F_{3, 47} = 2.05$, $P > .05$). Neither the VEH, 1 mg/kg phentolamine, nor 2.5 mg/kg phentolamine injection doses resulted in significant increases in PWTs ($P > .05$). However, the time effect was significant because the 5 mg/kg phentolamine significantly increased in PWT after the drug as compared with before the drug ($P < .01$). Fig 2B shows the von Frey thresholds of 7-day CPIP rats before and 20 minutes after treatment with VEH or 1, 2.5, and 5 mg/kg phentolamine. Two-way ANOVA reveals nonsignificant main effects of time (pre-post) ($F_{1, 47} = 1.36$, $P > .05$) and dose ($F_{3, 47} = 0.52$, $P > .05$) as well as a nonsignificant dose × time interaction ($F_{3, 47} = 0.43$, $P > .05$). Therefore, it appears that phentolamine is not effective at 7 days after reperfusion, and only the highest dose at 2 days after reperfusion is able to reduce mechanical allodynia in CPIP rats.

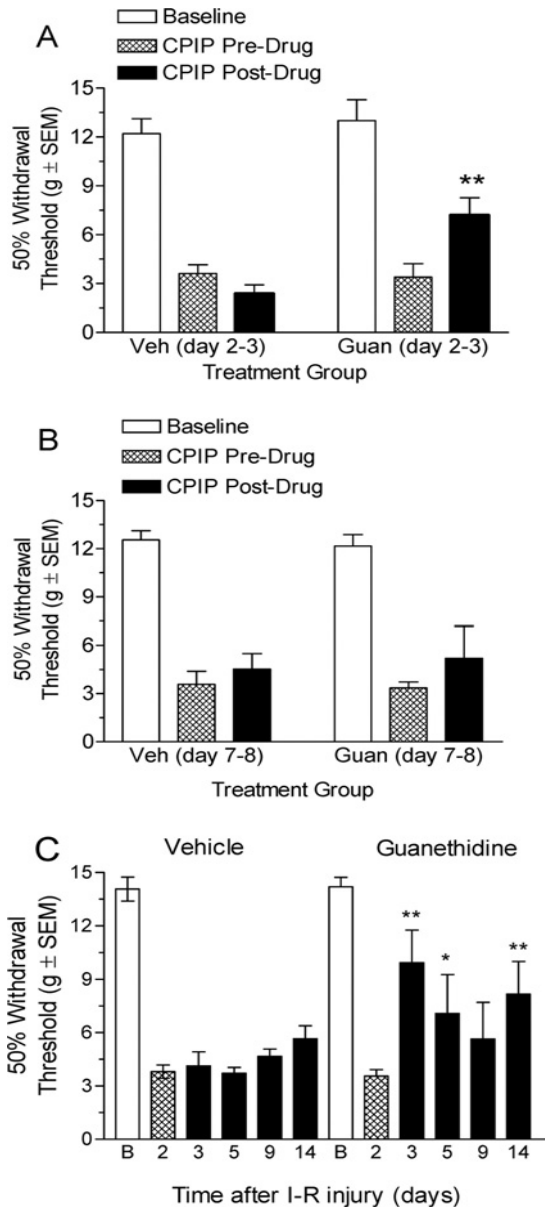

Figure 1. A, Effect of sympathetic block with guanethidine on mechanical allodynia in CPIP rats at 3 days after reperfusion. CPIP rats display a significant reduction in mechanical allodynia after 2-day guanethidine treatment starting 2 days after reperfusion (***P* < .01, as compared with CPIP before drug; n = 6 for each group). **B,** Effect of sympathetic block with guanethidine on mechanical allodynia in CPIP rats at 8 days after reperfusion. CPIP rats did not display a significant reduction in mechanical allodynia after 2-day guanethidine treatment starting 7 days after reperfusion (n = 6 for each group). **C,** Effect of sympathetic block with guanethidine on mechanical allodynia in CPIP rats at 3, 5, 9, and 14 days after reperfusion. CPIP rats display a significant reduction in mechanical allodynia at 3, 5 and 14 days, after 2-day guanethidine treatment starting 2 days after reperfusion (**P* < .05, ***P* < .01, as compared with CPIP before drug; n = 6 for guanethidine group, n = 7 for VEH group).

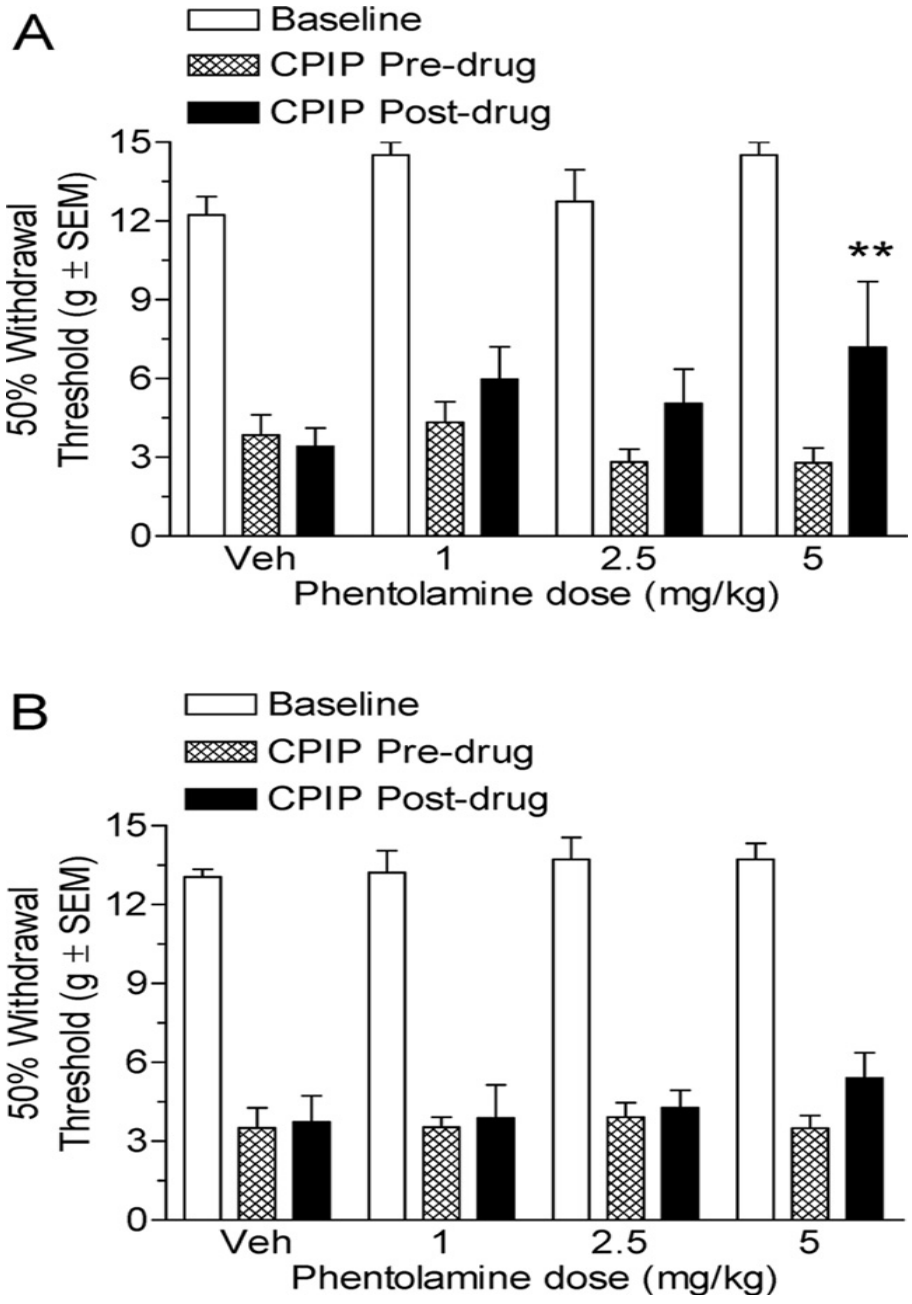

Figure 2. A, Effect of systemic phentolamine in CPIP rats at 2 days after reperfusion. CPIP rats display a significant reduction in mechanical allodynia with 5 mg/kg i.p. phentolamine administration but not with the 1 or 2.5 mg/kg doses (***P* < .1 as compared with CPIP before drug; n = 6 for each group). **B,** Effect of systemic phentolamine in CPIP rats at 7 days after reperfusion. CPIP rats did not display a significant reduction in mechanical allodynia with either 1, 2.5, or 5 mg/kg phentolamine doses at 7 days after reperfusion (n = 6 for each group).

3.4.3 Effect of the α_1-adrenergic antagonist prazosin on CPIP mechanical allodynia

Fig 3A shows the von Frey thresholds of 2-day CPIP rats before and 20 minutes after treatment with VEH or 1, 2.5, and 5 mg/kg prazosin. Two-way ANOVA reveals significant main effects of time (pre-post) ($F_{1, 47} = 28.27$, $P < .0001$) and dose ($F_{3, 47} = 3.90$, $P < .05$) and a significant time × dose interaction ($F_{3, 47} = 4.59$, $P < .05$). The VEH injection did not result in a significant increase in PWT ($P > .05$). All 3 prazosin doses, 1, 2.5, and 5 mg/kg, resulted in significant increases in PWTs ($P < 0.01$). Fig 3B shows the von Frey thresholds of 7-day CPIP rats before and 20 minutes after treatment with VEH or 1, 2.5, and 5 mg/kg prazosin. Two-way ANOVA reveals a significant main effect of time (pre-post) ($F_{1, 47} = 8.80$, $P < .01$), a nonsignificant main effect of dose ($F_{3, 47} = 0.67$, $P > .05$), and a nonsignificant time × dose interaction ($F_{3, 47} = 0.95$, $P > .05$). The VEH injection did not result in a significant increase in PWT ($P > .05$). The time effect was significant because the 2.5 mg/kg dose significantly increased the PWTs after the drug as compared with before the drug ($P < .05$). However, neither the 1 mg/kg nor the 5 mg/kg prazosin doses were able to significantly increase the PWTs relative to predrug levels ($P > .05$). However, rats that received the 5 mg/kg dose of prazosin did have significantly higher von Frey thresholds than rats that received VEH ($P < .05$). It appears that prazosin is able to reduce mechanical allodynia at both 2 and 7 days after reperfusion, and is more effective at 2 days after reperfusion in CPIP rats.

3.4.4 Effect of the α_2-adrenergic antagonist yohimbine on CPIP mechanical allodynia

Fig 4A shows the von Frey thresholds of 2-day CPIP rats before and 20 minutes after treatment with VEH or 1, 2.5, and 5 mg/kg yohimbine. Two-way ANOVA shows nonsignificant main effects of time (pre-post) ($F_{1, 47} = 0.61$, $P > .05$) and dose ($F_{3, 47} = 0.01$, $P > .05$) as well as a nonsignificant time × dose interaction ($F_{3, 47} = 1.02$, $P > .05$). This suggests that none of these doses of yohimbine are able to relieve mechanical allodynia at 2 days after reperfusion. Fig 4B shows the von Frey thresholds of 7-day CPIP rats before and 20 minutes after

treatment with VEH or 1, 2.5, and 5 mg/kg yohimbine. Two-way ANOVA shows nonsignificant main effects of time (pre-post) ($F_{1, 47} = 0.56$, $P > .05$) and dose ($F_{3, 47} = 0.66$, $P > .05$) as well as a nonsignificant time × dose interaction ($F_{3, 47} = 0.19$, $P > .05$). Therefore, it appears that yohimbine is unable to reduce mechanical allodynia in CPIP rats either at 2 days or 7 days after reperfusion.

3.4.5 Effect of the α_2-adrenergic agonist clonidine on CPIP mechanical allodynia

Fig 5A shows the von Frey thresholds of 2-day CPIP rats before and 20 minutes after treatment with VEH or 0.01, 0.025, and 0.1 mg/kg clonidine, as well as pretreatment with a 5 mg/kg yohimbine dose before a 0.1 mg/kg clonidine injection. Two-way ANOVA reveals a significant main effect of time (pre-post) ($F_{1, 59} = 12.34$, $P < .01$), a nonsignificant main effect of dose ($F_{4, 59} = 2.39$, $P > .05$), and a significant time × dose interaction ($F_{4, 59} = 3.55$, $P < .05$). Neither the VEH injection nor the 0.01 mg/kg clonidine dose resulted in a significant increase in PWT ($P > .05$). Both the 0.025 mg/kg and the 0.1 mg/kg clonidine doses resulted in significant increases in PWTs ($P < .05$ and $P < .01$, respectively). Further, pretreatment with yohimbine fully inhibited any increase in PWT observed with the highest dose of clonidine ($P > .05$), confirming that the effects are α_2-adrenergic receptor mediated. Fig 5B shows the von Frey thresholds of 7-day CPIP rats before and 20 minutes after treatment with VEH or 0.01, 0.025, and 0.1 mg/kg clonidine. Two-way ANOVA shows a significant main effect of time (pre-post) ($F_{1, 47} = 9.34$, $P < .01$), a nonsignificant main effect of dose ($F_{3, 47} = 1.1$, $P > .05$), and a nonsignificant time × dose interaction ($F_{3, 47} = 0.97$, $P > .05$). Neither the VEH injection, the 0.01 mg/kg, nor the 0.025 mg/kg clonidine doses resulted in significant increases in PWT ($P > .05$). The time effect was significant, however, since the 0.1 mg/kg clonidine dose significantly increased in PWTs after the drug as compared with before the drug ($P < .05$). Therefore, it appears that clonidine is able to significantly reduce mechanical allodynia at 2 days, and lesser so at 7 days, after reperfusion.

3.4.6 Time course of antiallodynia of adrenergic agents on CPIP

Fig 6 shows an antiallodynia time course of the highest dose of the above drugs (except guanethidine) on 2-day CPIP rats at 20, 40, 60, 80, 120, and 180 minutes after drug injection. Two-way ANOVA revealed significant main effects of group ($F_{4, 209} = 14.40$, $P < .0001$) and time ($F_{6, 209} = 13.29$, $P < .0001$) as well as a significant group × time interaction ($F_{24, 209} = 2.92$, $P < .0001$). None of the postinjection PWTs in the VEH group are significantly different from the preinjection PWT. For phentolamine, PWTs are significantly increased compared with VEH at the 60-minute time point ($P < .05$). For prazosin, PWTs are significantly increased compared with VEH at the 20-, 40-, 60-, 80- ($P < .01$), and 120-minute ($P < .05$) timepoints. For yohimbine, paw-withdrawal thresholds fail to differ significantly from VEH at any time point. Finally, for clonidine, PWTs are significantly increased compared with VEH at the 20-, 40-, 60-, 80- ($P < .01$), and 120-minute ($P < .05$) time points. Therefore, phentolamine, prazosin, and clonidine, but not yohimbine, relieve mechanical allodynia with peak antiallodynia at approximately 60 minutes after injection.

3.4.7 Effect of a nitric oxide donor vasodilator (SIN-1) on CPIP mechanical allodynia

Fig 7A shows the von Frey thresholds of 2-day CPIP rats before and 20 minutes after treatment with VEH or 1, 3, and 10 mg/kg of SIN-1. Two-way ANOVA revealed a significant main effect of time ($F_{1, 53} = 24.02$, $P < .0001$), a nonsignificant main effect of dose ($F_{3, 53} = 2.54$, $P > .05$), and a significant time × dose interaction ($F_{3, 53} = 4.87$, $P < .01$). The VEH injection did not result in a significant increase in PWT ($P > .05$). All 3 doses, 1, 2.5 ($P < .05$), and 10 mg/kg ($P < .01$), resulted in significant increases in PWTs. Fig 7B shows the von Frey thresholds of 7-day CPIP rats before and 20 minutes after treatment with VEH or 1, 3, and 10 mg/kg of SIN-1. Two-way ANOVA revealed nonsignificant main effects of time ($F_{1, 47} = 2.75$, $P > 0.05$) and dose ($F_{3, 47} = 1.05$, $P < .05$) as well as a nonsignificant time × dose interaction ($F_{3, 47} = 2.89$, $P > .05$). Therefore, SIN-1 is able to reduce mechanical allodynia at 2 days but not at 7 days after reperfusion in CPIP rats.

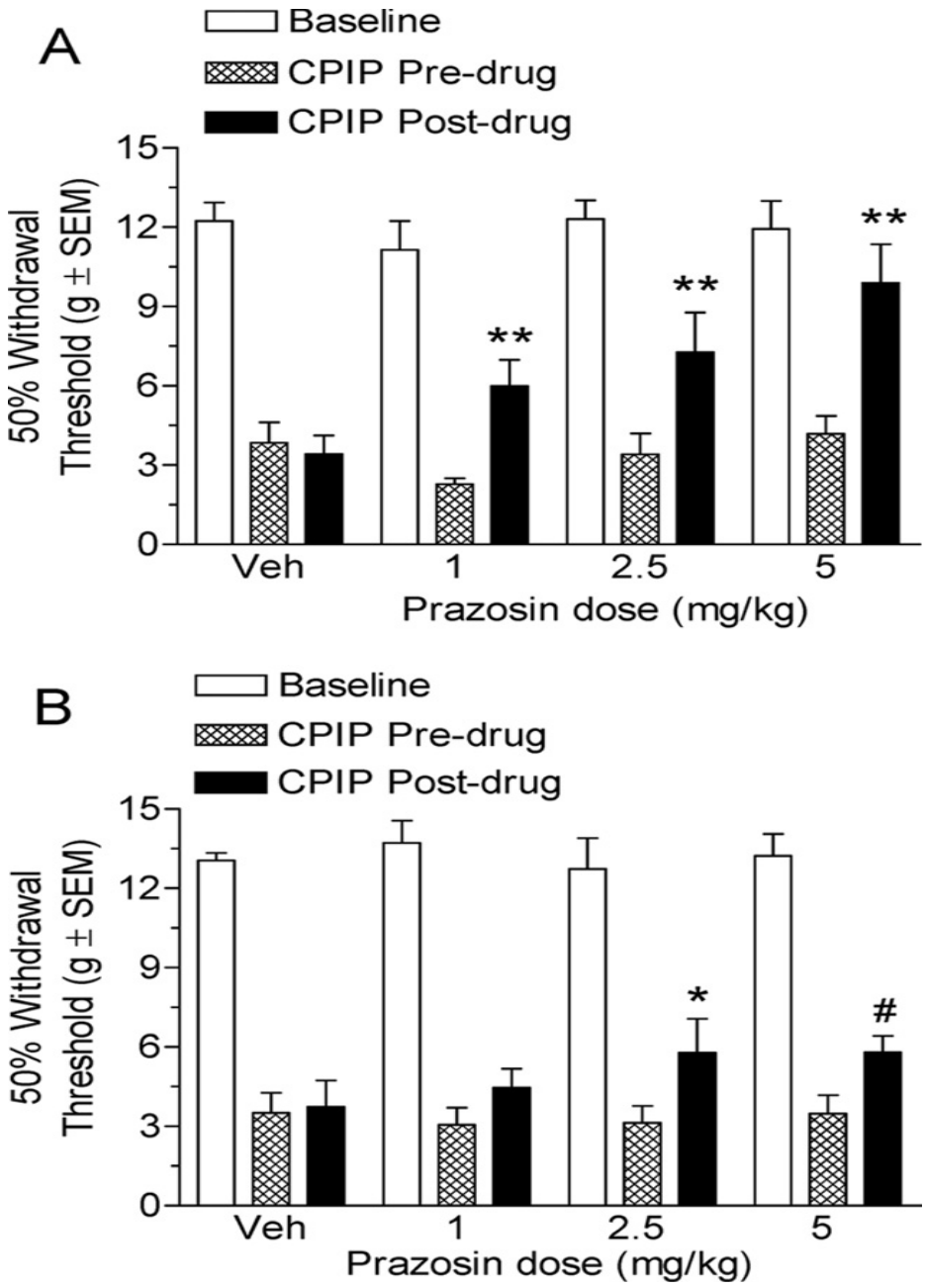

Figure 3. A, Effect of systemic prazosin in CPIP rats at 2 days after reperfusion. CPIP rats display a significant reduction in mechanical allodynia with 1, 2.5, and 5 mg/kg prazosin administration (***P* < .01 as compared with CPIP before drug; n = 6 for each group). **B,** Effect of systemic prazosin in CPIP rats at 7 days after reperfusion. CPIP rats display a significant reduction with the 2.5 and 5 mg/kg doses, but not the 1 mg/kg prazosin dose at 7 days after reperfusion (**P* < .05 as compared with CPIP before drug, #*P* < .05 as compared with CPIP after VEH; n= 6 for each group).

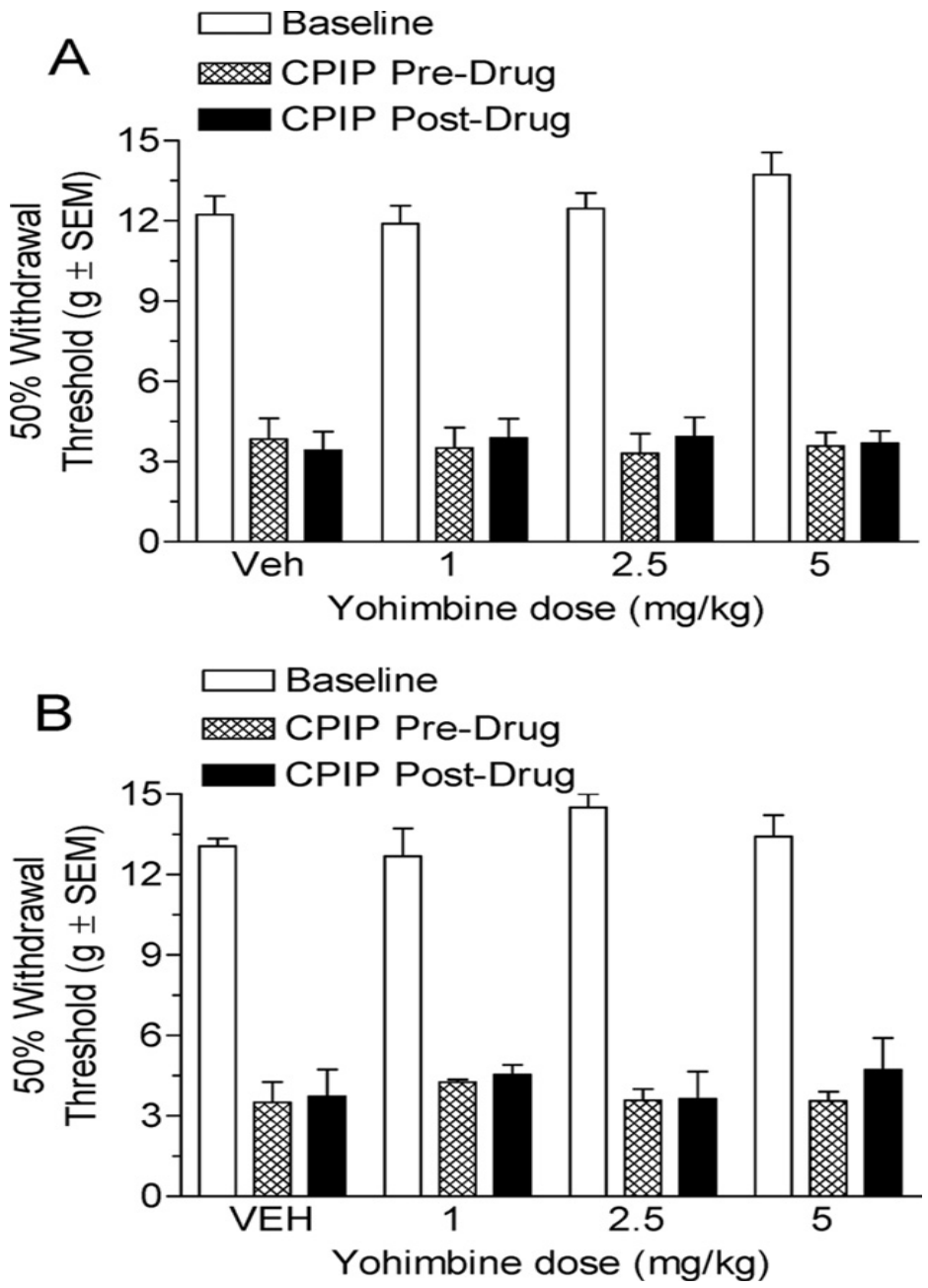

Figure 4. A, Effect of systemic yohimbine in CPIP rats at 2 days after reperfusion. CPIP rats did not display a significant reduction in mechanical allodynia with either the 1, 2.5, or 5 mg/kg yohimbine dose (n = 6 for each group). **B,** Effect of systemic yohimbine in CPIP rats at 7 days after reperfusion. CPIP rats did not display a significant reduction in mechanical allodynia with either 1, 2.5, or 5 mg/kg yohimbine dose at 7 days after reperfusion (n = 6 for each group).

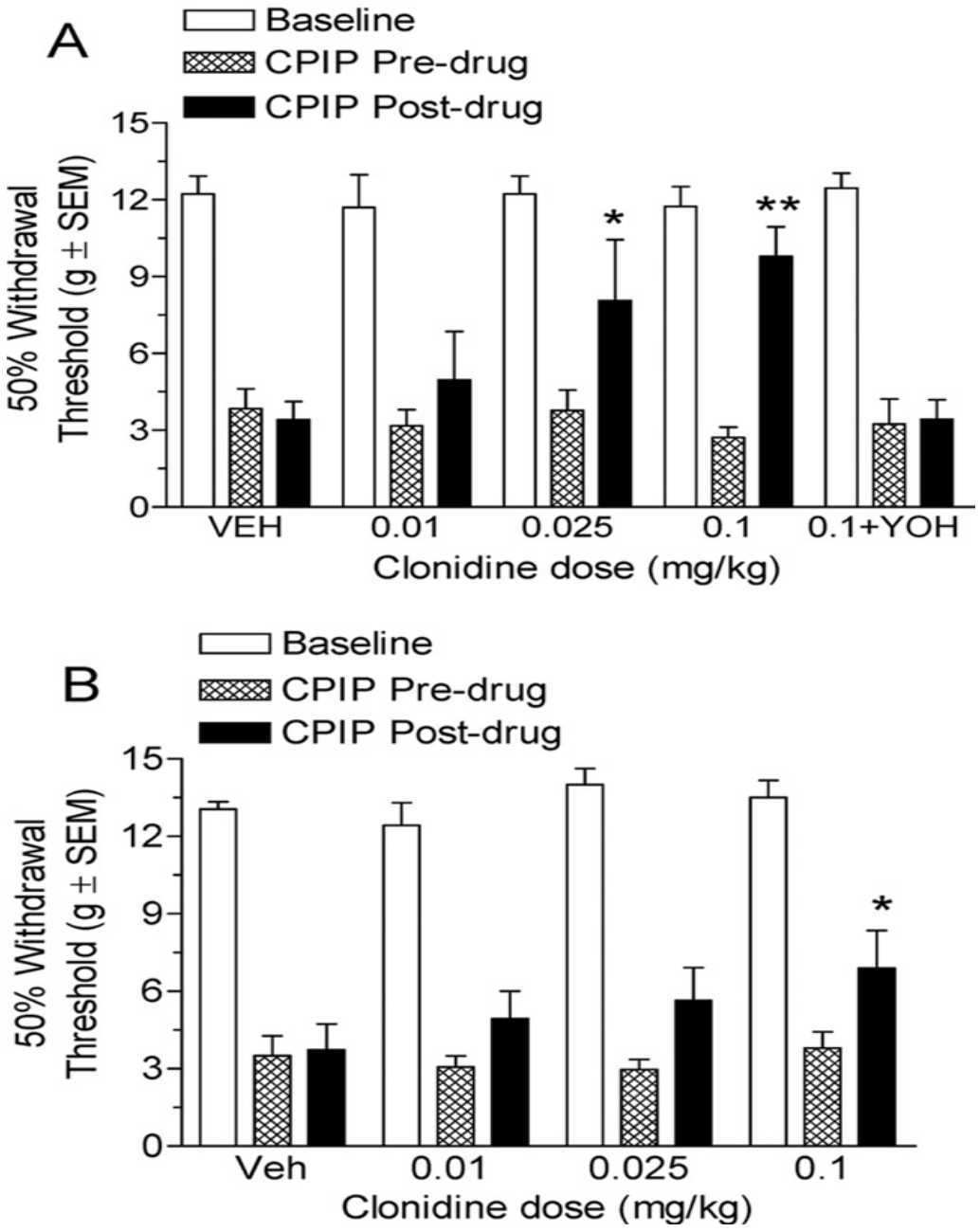

Figure 5. A, Effect of systemic clonidine in CPIP rats at 2 days after reperfusion. CPIP rats display significant reductions in mechanical allodynia with the 0.025 and 0.1 mg/kg clonidine doses but not with the 0.01 mg/kg dose. The effect of the highest dose of clonidine was reversed by yohimbine (**P* < .05 and ***P* < .01, as compared with CPIP before drug; n = 6 for each group). **B,** Effect of systemic clonidine in CPIP rats at 7 days after reperfusion. CPIP rats display a significant reduction in mechanical allodynia with a 0.1 mg/kg clonidine dose but not with the 0.01 or 0.025 mg/kg dose at 7 days after reperfusion. (**P* < .05 as compared with CPIP before drug; n = 6 for each group).

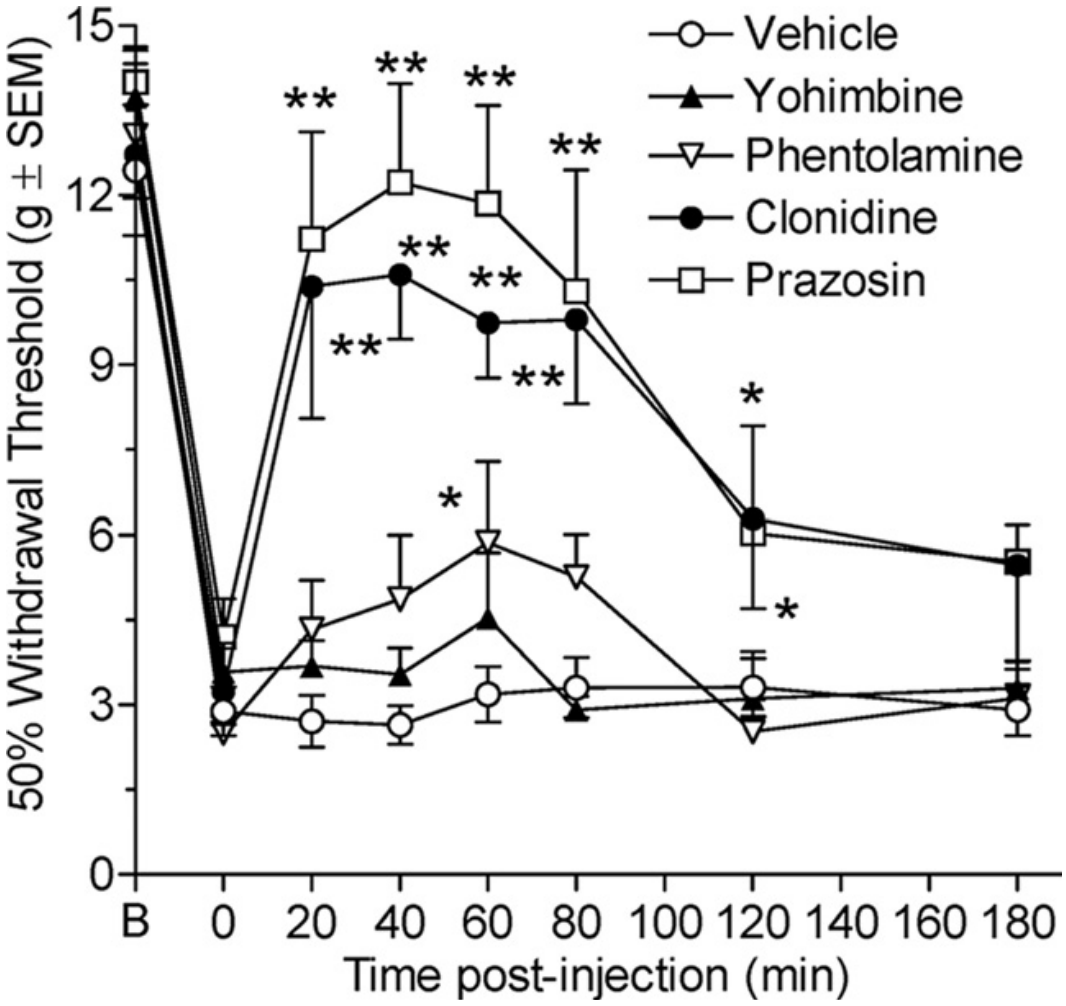

Figure 6. Three-hour time course of antiallodynic effects produced by systemic phentolamine, prazosin, yohimbine, and clonidine in 2-day CPIP rats. Phentolamine (5 mg/kg) significantly reduced mechanical allodynia at 60 minutes after injection. Prazosin (5 mg/kg) significantly reduced mechanical allodynia at 20, 40, 60, 80, and 120 minutes after injection. Yohimbine (5 mg/kg) did not significantly reduce mechanical allodynia at any time measured. Clonidine (0.1 mg/kg) significantly reduced mechanical allodynia at 20, 40, 60, 80, and 120 minutes after injection (*$P < .05$ and **$P < .01$, as compared with corresponding VEH time point; n = 6 for each group).

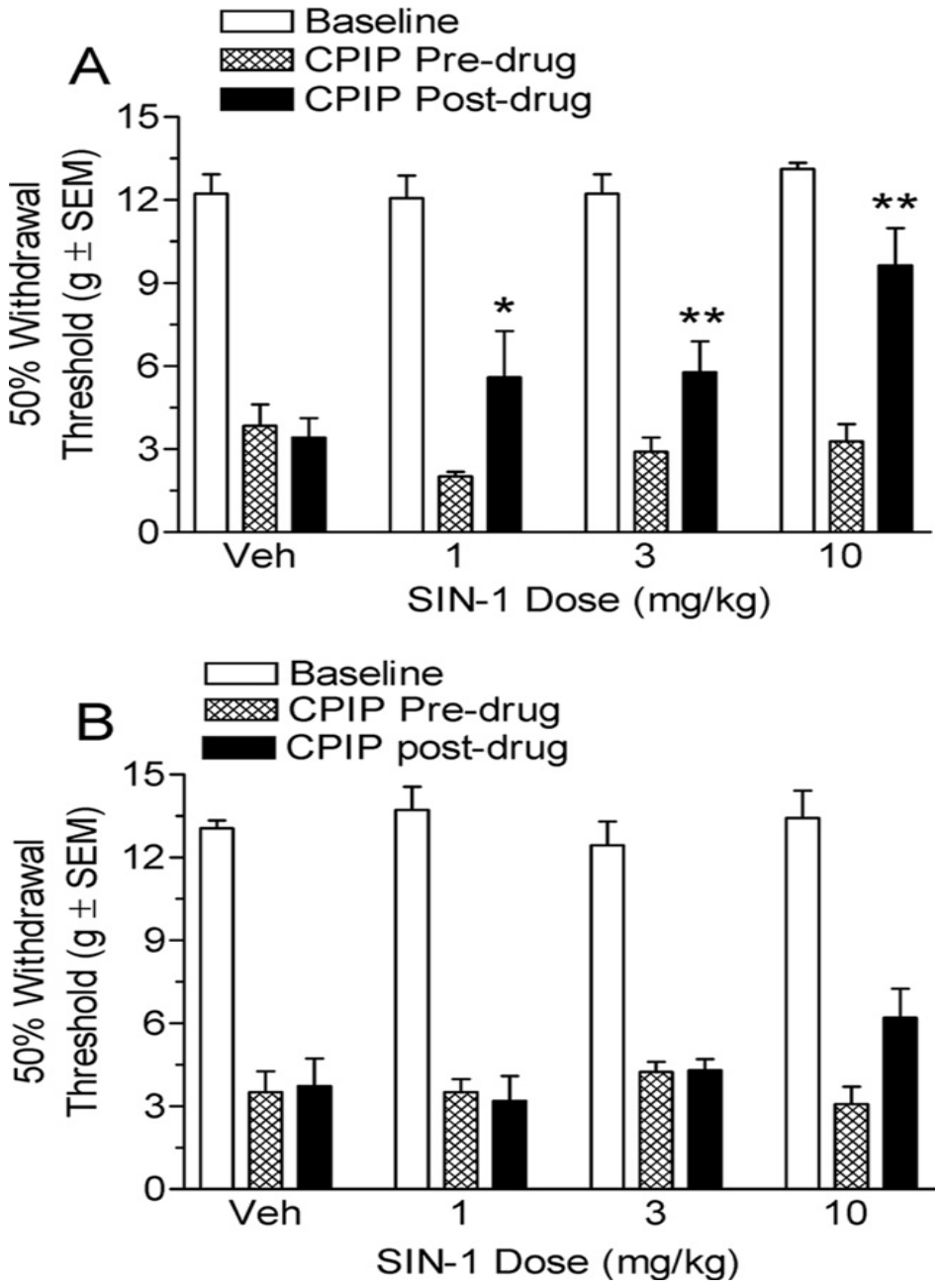

Figure 7. A, Effect of systemic SIN-1 in CPIP rats at 2 days after reperfusion. CPIP rats display significant reductions in mechanical allodynia with either 1, 3, or 10 mg/kg doses (**P* < .05 and ***P* < .01, as compared with CPIP before drug; n = 7 for each group). **B,** Effect of systemic SIN-1 in CPIP rats at 7 days after reperfusion. CPIP rats did not display significant reductions in mechanical allodynia with either 1, 3, or 10 mg/kg of SIN-1 at 7 days after reperfusion (n = 6 for each group).

3.5 Discussion

A contribution of the sympathetic system to pain mechanisms has been hypothesized in a variety of animal models of nerve injury and inflammation. Clinically, it has been suggested that it could be useful to consider subtypes of CRPS-I classified as SMP and SIP (Roberts, 1986; Campbell et al., 1998). However, the effectiveness of sympathectomy and sympathetic blockade as standardized treatment for CRPS has been questioned (Kingery, 1997; Mailis & Furlan, 2003). In animal models, sympathectomy has been shown to partially reduce mechanical allodynia after CCI, PSNL, and SNL (Shir & Seltzer, 1991; Kim et al., 1993; Kim et al, 1997; Malmberg & Basbaum, 1998). In CPIP rats, we found that sympathetic block with guanethidine partially reduced mechanical allodynia for up to 14 days when administered on days 2 and 3 after reperfusion; however, this effect was not significant when guanethidine was administered on days 7 and 8 after reperfusion. This is consistent with the clinical literature in which sympathetic blocks or sympathectomy may only be partially effective for the overall CRPS population, but also that they are particularly more effective early in the disease (Bonica, 1990; Singh et al., 2003).

Systemic administration of the nonspecific α-adrenergic antagonist phentolamine also partially reduces mechanical allodynia in CPIP rats at 2 days after reperfusion but, again, not significantly at 7 days after reperfusion. Along with sympathetic blocks, the response to phentolamine has been used clinically to identify SMP (Raja et al., 1991). Our results also resemble those in studies using rat SNL, in which both sympathectomy and systemic phentolamine reduce pain behaviours (Kim et al., 1993; Xie et al., 2001b), but this has not been consistently demonstrated (Ringkamp et al., 1999a; Ringkamp et al., 1999b). It has been suggested that for CRPS patients, the response to phentolamine predicts response to sympathetic blocks (Wehnert et al., 2002) and regional guanethidine (Arnér, 1991). CPIP rats are similar to CRPS patients because their allodynia is relieved both by phentolamine and guanethidine. Phenoxybenzamine, another nonselective α_1 antagonist, is also effective in CRPS (Muizelaar et al., 1997), although we did not study it here.

We found that the α_1- adrenergic antagonist prazosin, but not the α_2-antagonist yohimbine, effectively reduces mechanical allodynia, with almost a complete reversal for the 5 mg/kg prazosin dose in 2-day CPIP rats. This strongly suggests that the α_1-receptor contributes to the mechanical allodynia in CPIP. Lee et al. (1999) found that α_1-adrenergic antagonists, but not α_2-adrenergic antagonists, also reduce mechanical allodynia in rats with segmental spinal injury. Phentolamine and α_1- but not α_2-adrenergic antagonists also reduce cold allodynia in rats with S1/S2 SNL (Kim et al., 1993). Heat hyperalgesia in CCI rats is also reduced by α_1-adrenergic antagonists (Hord et al., 2001). Therefore, it appears that the CPIP model of CRPS-I displays similarities to animal models of CRPS-II, with each showing characteristics of SMP, and a critical role for α_1-receptors in SMP. As for the clinical use of these agents in CRPS, there are no carefully controlled trials with selective α_1- or α_2-adrenergic antagonists (Rowbotham, 2006).

The finding that yohimbine did not produce antiallodynia in our model and has even been found to exacerbate cold allodynia in rats with SNL (Kim et al., 2005a) suggests that antagonists of α_2-adrenergic receptors may have detrimental effects rather than beneficial ones in these models. Since many α_2-adrenergic receptors are autoreceptors mediating negative feedback on NE release from SPGN (Kahan, 1987; Saelens & Williams, 1983), α_2-antagonists are known to enhance SPGN NE release (Langer, 1980). The enhanced NE release would increase vasoconstriction, which we expect would be detrimental in CPIP rats, which already have poor blood flow (Laferrière et al., 2007). In a similar fashion, the action of phentolamine as an α_2-adrenergic antagonist may also be reducing its antiallodynic actions at the α_1-adrenergic receptor, providing a possible explanation for why phentolamine is less effective than prazosin in CPIP rats.

Like prazosin, systemic clonidine is particularly effective in CPIP rats at doses that do not produce defects on the rotorod (see Appendix B). Studies have shown that systemic clonidine can produce analgesic effects in humans (Hall et al., 2001) and topical administration relieves SMP in CRPS patients (Davis et al.,

1991). Systemic clonidine also partially relieves mechanical allodynia in rodents with CCI of the sciatic nerve (Kayser et al., 1995), and topical application has also been shown to be antinociceptive in animal studies (Dogrul & Uzbay, 2004). It is tempting to speculate that the analgesic effects of clonidine in CPIP rats are based on its known negative feedback effects on NE release from SPGNs (Kahan, 1987; Saelens & Williams, 1983). The α_2-agonist clonidine may also be particularly effective due to a combination of spinal analgesic and local vasodilatory effects (Figueroa et al, 2001; Hermann et al., 2005).

We have previously shown that the I-R injury associated with CPIP produces persistent tissue ischemia, indicated by reduced muscle perfusion (Laferrière et al., 2007), as well as vasoconstrictor hyper-responsiveness, indicated by an enhanced reduction in hind paw blood flow after close arterial injection of NE (Xanthos et al., 2008). The results here are consistent with a vasoactive role for NE from sympathetic efferents, in which activity at α_1-receptors is pronociceptive due to vasoconstriction in already ischemic tissue. Blocking the α_1-receptor with prazosin, therefore, results in vasodilatation and pain relief.

In the CPIP rats, we hypothesize that the I-R injury results in persistent tissue ischemia that contributes to the pain. Some of the known changes of I-R injury include vascular abnormalities such as persistent ischemia, dependent on either no-reflow due to capillary clogging (Blaisdell, 2002), or arterial vasospasms due to sympathetic vasoconstrictor hyper-responsiveness and/or endothelial cell dysfunction. Indeed, after I-R injury, there is both an upregulation and hyper-responsiveness of vascular adrenoceptors (Sapienza et al., 1996) as well as a reduced production and vasodilatory function of NO (Rubin et al., 1996; Khanna et al., 2005).

If persistent tissue ischemia contributes to CPIP pain (Laferrière et al., 2007), then CPIP pain should also be relieved by agents that enhance vasodilatation. In this study, we found that allodynia in CPIP rats was dose-dependently reduced by systemic administration of the NO donor SIN-1. We propose that by increasing the levels of NO, SIN-1 induces vasodilatation that

relieves pain-producing vasospasms and ischemia in CPIP rats. Indeed, it has been shown that blood flow can be improved in ischemic tissue with exogenous NO (Wang et al., 1997; Meldrum et al., 1999). Further, our results show that anti-allodynic doses of SIN-1, as well as another NO donor (SNP), also attenuate NE-evoked pain in CPIP rats (Xanthos et al., 2008). Therefore, it seems likely that NO-mediated vasodilation can reduce persistent tissue ischemia, and this may be a useful treatment for SMP.

In the CPIP rats, we find that antisympathetic and vasodilatory drugs are more effective at 2 days after reperfusion rather than 7 days after reperfusion. The temporal reduction in effectiveness may depend partly on a shift in the reliance of persistent ischemia on arterial vasospasms to no-reflow, a phenomenon that develops quickly after prolonged ischemia or repeated I-R injury (Allen et al., 1995; Laferrière et al., 2007; Nanobashvili et al., 2003). In CRPS-I patients, a slower development of no-reflow may also result in chronic tissue ischemia and pain that is more resistant to relief by sympathetic blockers.

In conclusion, CPIP rats show evidence of SMP (as defined clinically), since their mechanical allodynia is relieved by both sympathetic block and systemic phentolamine treatment. Furthermore, the responses to prazosin, clonidine, and the NO donor SIN-1 demonstrates that pain relief in CPIP rats can be produced by agents that decrease sympathetic vasoconstriction or enhance vasodilatation. We conclude that SMP mechanisms may involve exaggerated sympathetically mediated vascular contractility and persistent tissue ischemia.

3.6 Acknowledgments

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Intervening Section 2:

Experiments revealed that treatment with s.c. guanethidine and i.p. phentolamine significantly reduced mechanical allodynia in CPIP. Regional guanethidine and systemic phentolamine are used clinically to relieve SMP and some clinical studies also suggest that anti-sympathetic treatments are most effective when initiated early after injury. Therefore, the results suggested to us that CPIP rats displayed evidence of SMP. Systemic administrations of the α_1-adrenergic antagonist prazosin and the α_2-adrenergic agonist clonidine, but not that of the α_2-adrenergic antagonist yohimbine, were also effective in reducing CPIP mechanical allodynia. Consistent with known deleterious consequences on vasculature resulting from I-R injury such as vasospasms and no-reflow, we hypothesized that the vasodilatory action of the drugs used may be a particularly relevant anti-nociceptive mechanism in CPIP. To test this hypothesis, we also tested a NO donor vasodilator and found that it also reduced mechanical allodynia at 2 days post-reperfusion, an effect not reported before in animal models of CRPS-II. These findings suggested to us that response to anti-sympathetics in CPIP may be related to relieving hyper-responsiveness to efferent sympathetic vasoconstriction that develops as a consequence of I-R injury.

The literature with experimental hindlimb I-R injury suggests vascular changes are particularly important events, including no-reflow, vasospasms, endothelial dysfunction, and altered vascular reactivity to vasoactive agents. CRPS-I patients also have prominent vascular abnormalities as evidenced by ischemia, blood flow and temperature changes in the affected limb. Abnormal sympathetic vasoconstrictor responses have also been documented in these patients and increased vascular contractility to NE in the affected limb. Several authors have suggested that limb adrenergic hypersensitivity may occur in CRPS-I, possibly by upregulation of adrenergic receptors or other peripheral changes. Various studies have demonstrated that NE injected into the ipsilateral limb evokes exaggerated pain in these patients, particularly those with SMP.

Therefore, the next aim was to mimick clinical studies assessing nociceptive behaviours in CPIP rats using low doses of NE. We also sought to

determine whether CPIP rats display vascular abnormalities in peripheral tissues subjected to I-R injury that may be linked to NE vasoactive actions. NE was administered to the ipsilateral paw of CPIP rats, and the parallels between CPIP mechanical allodynia, NE-evoked nociceptive behaviours, and NE-evoked decreases in hind paw blood flow were examined. Further, we sought to test whether a non-adrenergic vasoconstrictor, or a NOS inhibitor could mimick NE-induced nociception, and also whether α_1- and α_2-adrenergic receptor antagonists and a vasodilator would inhibit NE-evoked nociceptive effects.

In the experiments with CPIP rats performed in chapter 2 and 3, and by other members in our laboratory, we repeatedly found that a small but consistent proportion of rats subjected to I-R injury did not develop mechanical allodynia. In order not to test anti-allodynia in rats that did not have allodynia, these rats were excluded from pharmacological trials. However, in this study, we decided it would be interesting to use these rats to establish whether non-allodynic rats would also lack vasconstrictive NE hypersensitivity.

Chapter 4

Norepinephrine-induced nociception and vasoconstrictor hypersensitivity in rats with chronic post-ischemia pain

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4.1 Abstract

Painful hypersensitivity to NE has been reported in various chronic pain conditions that exhibit SMP, particularly CRPS-I and II. We investigated the parallels between the nociceptive and vascular sensitivity to NE in rats with CPIP, an animal model of CRPS-I induced by hind paw ischemia–reperfusion injury. Intradermal injections of NE to the affected hind paw induced dose-dependent nociceptive behaviours in CPIP rats, but not sham animals. These behaviours were blocked by α_1- and α_2-adrenergic receptor antagonists, or a NO donor. Using laser Doppler flowmetry, we detected vasoconstrictor hypersensitivity in the ipsilateral CPIP hind paw, as compared to responses in sham animals or the contralateral hind paw. The vasoconstrictor hypersensitivity was also attenuated by adrenergic antagonists. Intradermal injection of [Arg^8] vasopressin (AVP) or the eNOS inhibitor, (N5-(1-iminoethyl)-l-ornithine dihydrochloride (L-NIO), to the affected paw also induced nociceptive behaviours in CPIP rats, but not sham rats. These results suggest CPIP rats display abnormal nociceptive responses to adrenergic and non-adrenergic vasoconstrictive agents. Furthermore, the nociceptive responses to NE in CPIP rats are paralleled by enhanced vasoconstrictive responses to NE, and are relieved by α-adrenergic antagonists or a vasodilator. We conclude that persistent tissue ischemia and hypersensitivity to sympathetic vasoconstriction are important mechanisms for pain in CPIP rats, and that either reducing vasoconstriction or enhancing vasodilatation may be effective methods of relieving the pain of CRPS-I.

Keywords: Chronic post-ischemia pain; Adrenergic receptors; Ischemia; Laser Doppler; Vasoconstriction; [Arg^8] vasopressin; L-NIO; SNP; SIN-1; Complex regional pain syndrome; CRPS-I

4.2. Introduction

Intradermal injection of NE normally produces a burning, stinging pain that lasts tens of seconds. In patients with CRPS-I or II, intradermal NE (Torebjörk et al., 1995), or the α_1-adrenergic agonist phenylephrine (Mailis-Gagnon & Bennett, 2004), produces the same transient pain, but also abnormal burning pain and mechanical allodynia lasting for tens of minutes. Abnormal responses to NE or phenylephrine are also found in CRPS-I and II patients who have first had their pain relieved by surgical sympathectomy, sympathetic ganglion blocks or intradermal treatment with phentolamine or clonidine (Wallin et al., 1976; Davis et al., 1991; Torebjörk et al., 1995; Ali et al., 2000). The ability of intradermal NE to rekindle pain relieved by sympathectomy, or sympathetic blocks (Torebjörk et al., 1995), suggests that the pain depends more on hyper-responsiveness of adrenoceptors than on hyperactivity of sympathetic fibers.

The hypersensitivity to NE, along with the reduction of pain and allodynia in some CRPS-I and II patients with sympathetic blocks or adrenergic antagonists, has been taken as evidence for SMP (Roberts, 1986; Campbell et al., 1988). Importantly, exaggerated painful responses to NE are exhibited in CRPS-I and II patients whose pain is relieved by sympathetic blocks (i.e., SMP), but not in those whose pain is not relieved by sympathetic blocks or phentolamine (SIP) (Torebjörk et al., 1995; Ali et al., 2000; Mailis-Gagnon & Bennett, 2004).

While experimental evidence has suggested that SMP may depend on sympathetic efferent–primary afferent coupling (Chen et al., 1996) that causes *de novo* adrenergic sensitivity in damaged afferents (Wall & Gutnick, 1974; Devor & Jänig, 1981), DRGs (Devor et al., 1994; Michaelis et al., 1996) or nociceptors (Sato & Perl, 1991; O'Halloran & Perl, 1997), clinical evidence further suggests that SMP may depend on sympathetic-dependent vasoconstriction that produces pain by reducing blood flow in the affected tissue (Kurvers et al., 1995; Wasner et al., 2001a; Baron et al., 2002; Ackerman et al., 2005). Indeed, NE-induced pain in CRPS patients occurs at doses which produce vasoconstriction (Ali et al., 2000), and CRPS-I patients show a hyper-responsiveness of vascular responses to NE (Arnold et al., 1993; Birklein et al., 1997; Teasell & Arnold, 2004). Furthermore,

it has been shown that there is enhanced vasoconstriction to exogenous NE following CCI of the sciatic nerve (an animal model of CRPS-II) (Kurvers et al., 1997; Kurvers et al., 1998). Recently, our group developed a novel animal model of CPIP that is created by a 3-h hind paw tourniquet ischemia and displays persistent mechanical and cold allodynia as a result of an ischemia–reperfusion (I–R) injury (Coderre et al., 2004; Laferrière et al., 2007). The purpose of this study is to examine the relationship between NE-evoked nociception and vasoconstrictor hypersensitivity (as reflected by NE-induced changes in skin blood flow) in this animal model which may be particularly relevant to CRPS type I.

4.3 Methods

4.3.1 Animals

Male Long Evans hooded rats (275–325 g, Charles River, St. Constant, Que., Canada) were housed in groups of 3–4, with food and water available *ad libitum*, on a 12:12 h light:dark cycle. All treatments and testing procedures were approved by the Animal Care Committee at McGill University, and conformed to the ethical guidelines of the Canadian Council on Animal Care and the IASP.

4.3.2 Animal model of CRPS-I

CPIP was generated following exposure to prolonged hind paw ischemia and reperfusion as described in Coderre et al. (2004). Briefly, rats were anesthetised over a 3 h period with a sodium pentobarbital infusion. After induction of anesthesia, a Nitrile 70 Durometer O-ring (O-rings West, Seattle, WA, USA) with 7/32″ internal diameter was placed around the rat's left hind limb just proximal to the ankle joint. The termination of sodium pentobarbital anesthesia was timed so that rats recovered fully within 30–60 min following reperfusion, which occurred immediately after removal of the O-ring. Sham rats received exactly the same treatment, except that the O-ring was cut so that it only loosely surrounded the ankle, and did not occlude blood flow to the hind paw.

4.3.3 Drugs

Norepinephrine bitartrate dehydrate (NE), yohimbine, prazosin and SNP were all obtained from Sigma–Aldrich (St. Louis, MO, USA). [Arg^8] Vasopressin

(AVP) was obtained from Calbiochem (La Jolla, CA, USA). SIN-1 and *N*5-(1-iminoethyl)-l-ornithine dihydrochloride (L-NIO) were obtained from Tocris (Bristol, United Kingdom). All agents were diluted in a 0.9% saline VEH immediately before experiments.

4.3.4 Nociceptive testing

4.3.4.1. Mechanical sensitivity

Hind paw mechanical thresholds were assessed by measuring the withdrawal response to von Frey filament stimulation according to a modification of the up/down method described by Chaplan et al. (1994). In brief, animals were placed in a Plexiglas® box ($21 \times 16 \times 27$ cm^3) with a wire grid bottom through which the von Frey filaments (nylon monofilaments; Stoelting, Woodale, IL, USA) were applied to the plantar surface of the hind paw. Filaments were applied in either ascending or descending strength as necessary to determine the filament closest to the threshold of response. Each filament was applied once for 10 s to the center of the paw between the digital tori. A lower intensity hair followed each positive response and a higher intensity hair followed each negative response until five responses were recorded after a first change in response. The minimum filament intensity was 0.25 g and the maximum was 15 g. Based on the response pattern and the force of the final filament, the 50% response threshold (grams) was calculated. The resulting pattern of positive and negative responses was tabulated, and the 50% response threshold was interpolated using the formula: 50% g threshold = $(10^{[x_f + k\delta]})/10{,}000$, where x_f is the value (in log units) of the final von Frey hair used; k is tabular value (see Chaplan et al. (1994) for pattern of positive/negative responses); and δ is the mean difference (in log units) between stimuli (here 0.224). Hairs were from the standard Semmes–Weinstein series (Semmes et al., 1960). Mechanical sensitivity was tested before CPIP induction, and 2 and/or 7 days post-reperfusion. Animals were classified prior to experiments as responders if their von Frey paw withdrawal scores were below 6 g (65.4%) and non-responders if their PWT scores were above 10 g (27.3%). Animals with von Frey scores between 6 and 10 g (7.3%) were not used.

4.3.4.2. Evoked nociceptive behaviours

To measure pain evoked by intradermal injections, rats were placed in Plexiglas® boxes with a mirror underneath in order to observe nociceptive behaviours. Rats were habituated to the testing apparatus 30 min each day for 2–3 days prior to testing and for a minimum of 30 min immediately prior to testing. Drugs were injected in volumes of 20 μl to the plantar surface of the hind paw using a 26 G needle. Two injected rats were then observed simultaneously for 15 min, and the total time spent exhibiting hind paw stamping, elevation or licking was recorded. Experiments were performed in blocks with groups of sham rats, CPIP rats and CPIP non-responder rats tested on the same days. Rats were only used for one experiment, and at the time of testing the experimenter was blind to the animal's treatment.

In the first behavioural experiment, we assessed whether saline VEH, or 10, 50 or 250 ng of i.pl. NE-induced nociceptive behaviours in sham or 2- or 7-day CPIP responders and non-responders (n = 6–9/group). In subsequent experiments, adrenergic antagonists (0.5, 2 and 10 μg of prazosin and yohimbine) were co-injected with 250 ng NE to determine which adrenoceptors contributed to the NE-induced behaviours in 2-day CPIP rats (n = 6/group). The prazosin and yohimbine doses used here have been shown to relieve mechanical hyperalgesia in rat models of neuropathic pain (Tracey et al., 1995; Ringkamp et al., 1999b). Additional studies assessed whether NE-induced pain behaviours in 2-day CPIP rats were attenuated by i.pl. or systemic administration of NO donors. SNP was co-injected with 250 ng NE at doses of 20, 100 and 500 μg (n = 6/group), and systemic SIN-1 (10 m/kg) or VEH (n = 6–8) was used as a pretreatment prior to 250 ng i.pl. NE. The doses of the NO donors used here have previously been shown to reduce allodynia in a rat model of inflammatory pain (Da Rocha et al., 2002), and do not produce motor or sedative side effects (Xanthos & Coderre, 2008). Finally, we examined whether i.pl. injections of a non-adrenergic vasoconstrictor, AVP (500 ng), or a NOS inhibitor, L-NIO (250 μg), (n = 7–10 per group) induced nociceptive behaviours in sham or 2-day CPIP rats.

4.3.5 Laser Doppler flowmetry

Laser Doppler flowmetry was used to assess blood flow changes associated with either close arterial or i.v. injection of NE. After behavioural testing for von Frey thresholds, animals were anesthetised with urethane (1 mg/kg, i.p.), and their body temperature was maintained at 37 °C using a thermostatically-controlled heating pad coupled to a rectal probe (Stoelting, IL, USA). For close arterial injections of NE, the common iliac artery was exposed on the side contralateral to the CPIP injury. The artery was catheterized with PE-10 tubing filled with warm saline (37 °C) and aimed upwards towards the aortic bifurcation so that solutions were injected immediately upstream of the common iliac artery on the CPIP side. The wound was covered with saline-soaked gauze. A laser Doppler probe (DP1T-V2; Moor Instruments, Axminster, United Kingdom) with a height of 12.5 mm, outer diameter of 8 mm, and a fiber separation of 0.5 mm was taped loosely on the plantar surface of the ipsilateral paw adjusting the position and pressure to give initial readings of approximately 150–300 arbitrary flux units. Blood flow was recorded continuously with a DRT4 monitor (Moor Instruments, Axminster, United Kingdom). Animals were covered with a blanket and left undisturbed for approximately 60 min in order to obtain stable baseline for flux, as well as hind paw and core body temperatures. Animals whose baseline arbitrary flux units were below 150 were not used.

Since close arterial injection necessitated cannulating and compromising blood flow in the common iliac artery contralateral to the CPIP injury, it was not possible in these experiments to compare NE-induced changes in blood flow in the ipsilateral and contralateral hind paws. In order to make this comparison, we also performed laser Doppler flowmetry following i.v. administration of NE to the jugular vein. The jugular vein was catheterized with PE-20 tubing, and two separate laser Doppler probes were used to simultaneously record the ipsilateral and contralateral hind paw blood flow in CPIP rats.

4.3.5.1. NE-evoked vasoconstriction

All drugs and saline were warmed to 37 °C and 20 µl of drug/VEH was injected followed by an 80 µl saline flush. An initial saline (control) injection was given after establishment of a steady baseline. No flow disturbance was observed, and subsequently, ascending doses of NE (50, 100 and 200 µg/kg) were administered every 20 min to naive (n = 7), sham (n = 13), 2-day CPIP responders (n = 8), 7-day CPIP responders (n = 9), 2-day CPIP non-responders (n = 9), and 7-day CPIP non-responders (n = 7). Dose–response effects of close arterial NE injections were also examined in rats that were pretreated intra-arterially with 10 µg of either prazosin or yohimbine (n = 8 per group). The same doses of NE that were used for close-arterial injections were used in another experiment to compare the effect of i.v. NE on blood flow changes in the ipsilateral and contralateral hind paws of sham (n = 9) and 2-day CPIP responders (n = 9). Rats were only used once and were sacrificed at the end of the experiment with an overdose of sodium pentobarbital.

4.3.5.2 Flowmetry calculations

Baseline flux was defined as the average flux value in the 3 min prior to the initial injection (saline). Peak decreases in blood flow, expressed as % change from baseline, were calculated using the minimum flux value after each NE injection. Since arbitrary flux values are dependent on depth of anesthesia and laser Doppler probe placement, this study assessed only NE-induced changes in arbitrary flux values, and did not compare differences in basal flux values between groups. Although reduced basal flux might be expected after CPIP, and we have previously shown that there is reduced perfusion in muscle of the ipsilateral hind paw (Laferrière et al., 2007), we did not here observe ipsilateral/contralateral differences in baseline flux values in CPIP rats.

4.3.6 Statistics

Group comparisons of NE-evoked nociceptive scores were performed using two-way ANOVA followed by Fisher's LSD tests. Group comparisons for laser Doppler peak flux decreases were analyzed using two-way repeated

measures ANOVA and Fisher's LSD test. One-way ANOVA was used to compare the behavioural effects of SNP vs. VEH and AVP vs. VEHs.

4.4. Results

4.4.1. NE-induced nociceptive responses

Approximately 70% of animals subjected to the I–R injury displayed mechanical allodynia (von Frey threshold below 6 g) and were classified as responders. This is consistent with our past studies as reported in Coderre et al. (2004).

Fig. 1A shows the time spent exhibiting VEH- and NE-evoked nociceptive behaviours in sham rats at 2 days and CPIP rats (responders) at 2 and 7 days post-reperfusion. Nociceptive behaviours generally started a few seconds after injection and peaked between 2 and 5 min post-injection. The most common behaviours were hind paw stamping, elevation and licking. Two-way ANOVA revealed a significant main effect of group ($F_{2, 89} = 16.53$, $P < 0.0001$), and dose ($F_{3, 89} = 9.05$, $P < 0.0001$) and a significant group × dose interaction ($F_{6, 89} = 2.31$, $P < 0.05$) for animals given intradermal injections of VEH, 10, 50 or 250 ng of NE. The VEH injection did not produce significantly greater nociceptive behaviours in CPIP rats than in sham rats. However, at 2 days post-perfusion, CPIP rats given either 50 ($P < 0.05$) or 250 ng ($P < 0.01$) of NE exhibited significantly more nociceptive behaviours than CPIP rats given VEH. For the CPIP rats at 7 days post-reperfusion, the 50 and 250 ng doses of NE also produced significantly more nociceptive behaviours ($P < 0.01$) as compared to VEH in CPIP rats.

Fig. 1B shows the time spent exhibiting VEH- and NE-evoked nociceptive behaviours in sham rats and CPIP *non-responders* at 2 and 7 days post-reperfusion. Two-way ANOVA revealed a significant main effect of group ($F_{2, 80} = 11.87$, $P < 0.0001$), but a non-significant main effect of dose, and a non-significant group × dose interaction. Post hoc analysis revealed that once again sham rats did not exhibit greater response to any dose of NE relative to the VEH, and CPIP non-responders did not exhibit significantly more nociceptive behaviours to VEH than did sham rats. At 2 days post-reperfusion, none of the NE

doses induced significantly more nociceptive behaviours in CPIP non-responder as compared to CPIP non-responder rats injected with VEH. However, at 7 days post-reperfusion, the 50 and 250 ng doses of NE induced significantly more nociceptive behaviours in CPIP non-responders as compared to VEH injections in CPIP non-responders ($P < 0.05$ for the 50 ng dose and $P < 0.01$ for the 250 ng dose).

4.4.2 Hind paw blood flow responses to close arterial NE

Fig. 2 shows a sample trace for the laser Doppler protocol. The trace shows that flux levels are not altered in response to saline injection, but are lowered between 100 and 300 arbitrary units for durations that increase with greater intra-arterial doses of NE between 50 and 200 μg/kg.

Baseline arbitrary flux values were not significantly different between naive, sham, CPIP 2-day responders, CPIP 7-day responders, CPIP 2-day non-responders and CPIP 7-day non-responders. There were no significant group differences between naı¨ve and shams in their NE-evoked decreases in blood flow, so these groups were combined into a single control group. Although a range of doses were tested to elicit significant detectable decreases in flux values, we found that doses below 50 μg/kg showed inconsistent flux decreases in some animals, while doses greater than 200 μg/kg were subject to significant ceiling effects. The peak response was usually seen approximately 1–2 min post-injection, and the return to baseline occurred approximately 7–8 min post-injection for the 50 μg/kg dose, 13–14 min post-injection for the 100 μg/kg dose, and usually over 20 min post-injection for the 200 μg/kg (the experiment was stopped 20 min post-injection).

Fig. 3A shows dose–response curves for NE-evoked flux decreases in controls and CPIP rats at 2 and 7 days post-reperfusion. Two-way repeated measures ANOVA demonstrates a significant main effect for group ($F_{2, 113} = 5.28$, $P < 0.05$), a significant main effect of dose ($F_{2, 113} = 19.94$, $P < 0.01$) but a non-significant group × dose interaction. Except for the 50 μg/kg dose of the CPIP 7-day responder, post hoc analyses reveal that CPIP rats had significantly greater

peak decreases in flux compared to controls for all three NE doses at both 2 and 7 days post-reperfusion ($^{*}P < 0.05$, $^{**}P < 0.01$).

Fig. 3B shows dose–response curves for intra-arterial NE-induced decreases in blood flow for controls and CPIP *non-responders* at 2 and 7 days post-reperfusion. ANOVA reveals a significant main effect of dose ($F_{2, 107} = 22.22$, $P < 0.0001$), but a non-significant main effect of group, and a non-significant group × dose interaction. These results indicate that NE-induced blood flood decreases did not significantly differ between CPIP non-responders and control rats.

4.4.3 Effect of intravenous NE administration in CPIP responders

In the animals prepared with jugular vein cannulae, baseline arbitrary flux values were not significantly different between the ipsilateral and contralateral hind paws in CPIP responders ($n = 9$) and shams ($n = 9$). Fig. 3C shows dose–responses curves for i.v. NE-evoked decreases in blood flow for CPIP responders with simultaneous ipsilateral and contralateral hind paw recordings. There is a significant effect of side ($F_{1, 53} = 7.27$; $P < 0.05$), a significant effect of dose ($F_{2, 53} = 21.13$; $P < 0.0001$), and a non-significant side × dose interaction. Post hoc analyses reveal a significant difference between ipsilateral and contralateral paws with the 50 and 100 μg/kg NE doses ($^{*}P < 0.05$, $^{**}P < 0.01$). Fig. 3D shows dose–response curves for i.v. NE-evoked decreases in blood flow for shams with simultaneous ipsilateral and contralateral hind paw recordings. Although there was a significant effect of dose ($F_{2, 53} = 35.12$; $P < 0.0001$), there was a non-significant effect of side and a non-significant side × dose interaction.

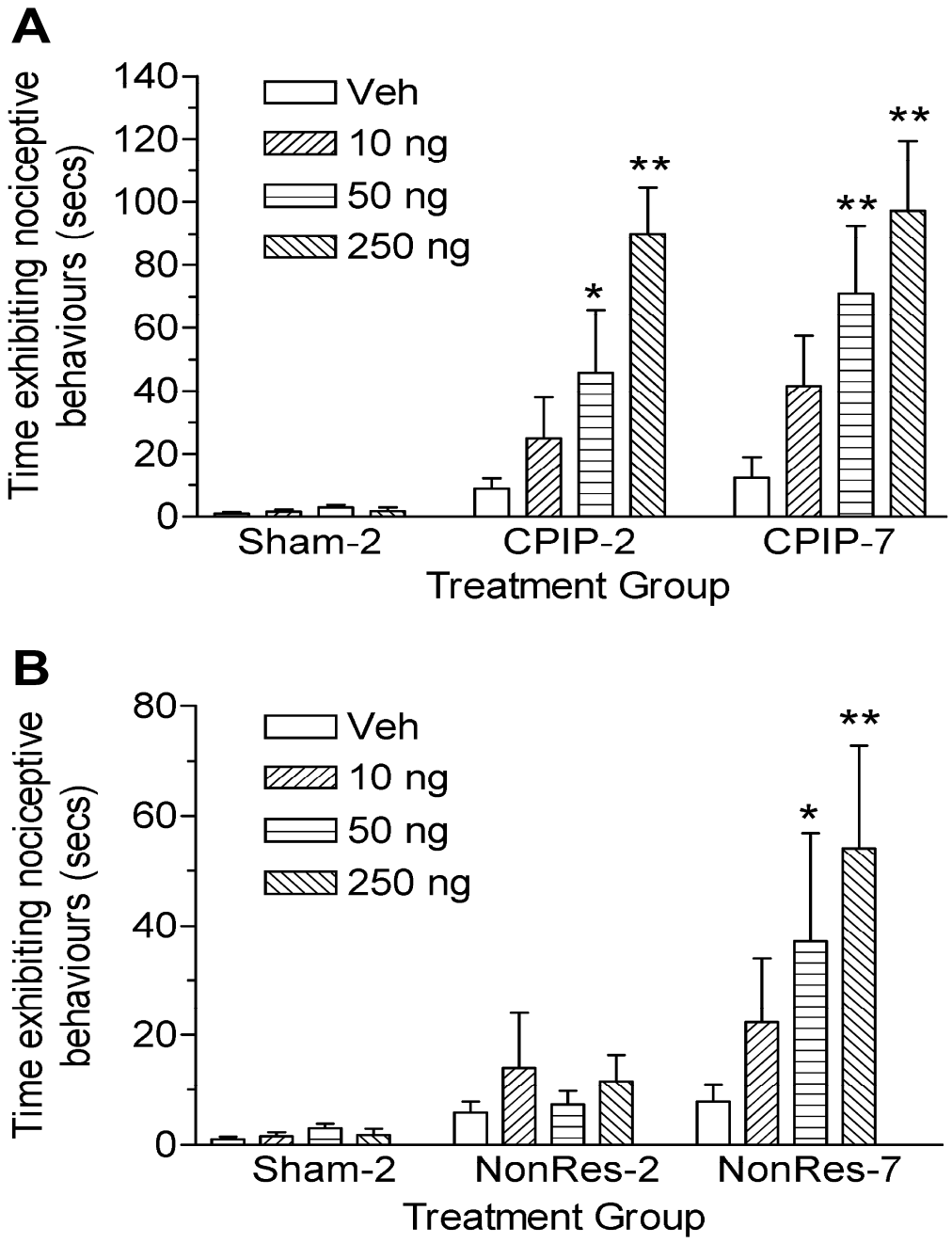

Figure 1. (**A**) Intradermal NE-evoked nociception in CPIP responders at 2 and 7 days post-reperfusion. CPIP responders displayed exaggerated NE-induced nociception with the 50 and 250 ng NE doses on days 2 and 7. ($^{*}P < 0.05$ and $^{**}P < 0.01$). (**B**) Intradermal NE-evoked nociception in CPIP non-responders at 2 and 7 days post-reperfusion. CPIP non-responders displayed exaggerated NE-induced nociception with the 50 and 250 ng NE doses on day 7 only. ($^{*}P < 0.05$ and $^{**}P < 0.01$)

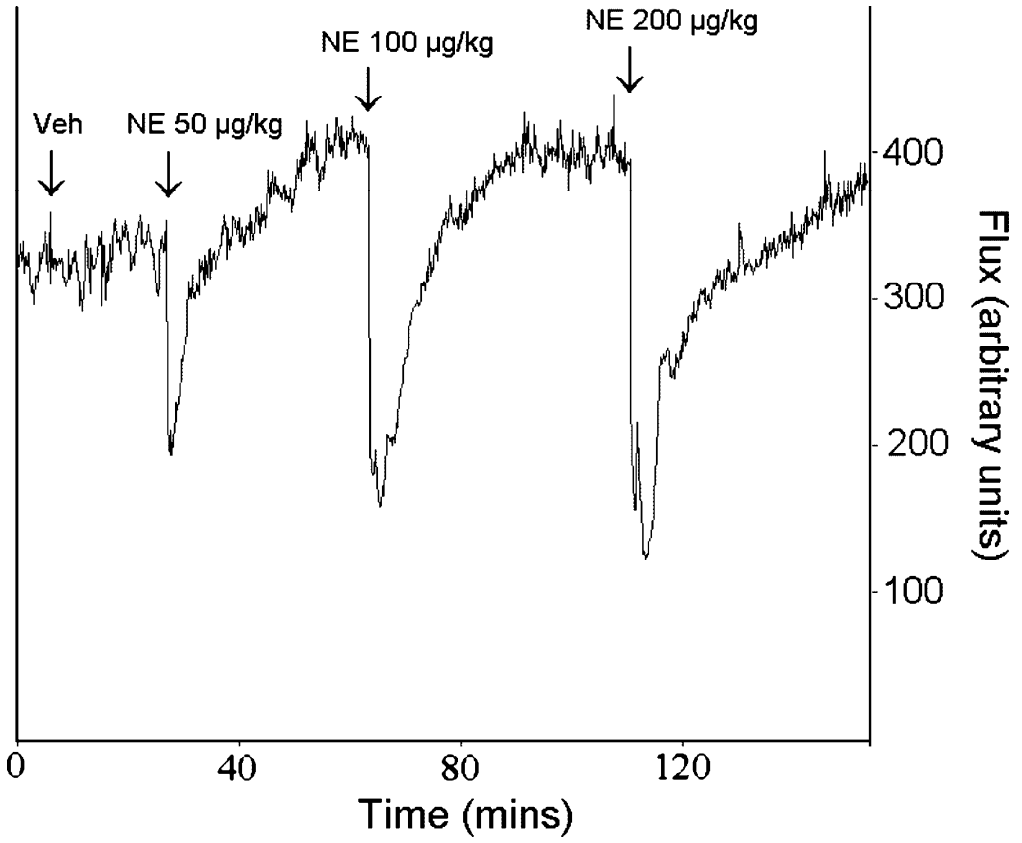

Figure 2. Sample laser Doppler trace showing an NE dose–response curve in a naive rat. Intra-arterial saline results in no alteration in blood flow. Intra-arterial NE produces dose-dependent decreases in blood flow (arbitrary flux units).

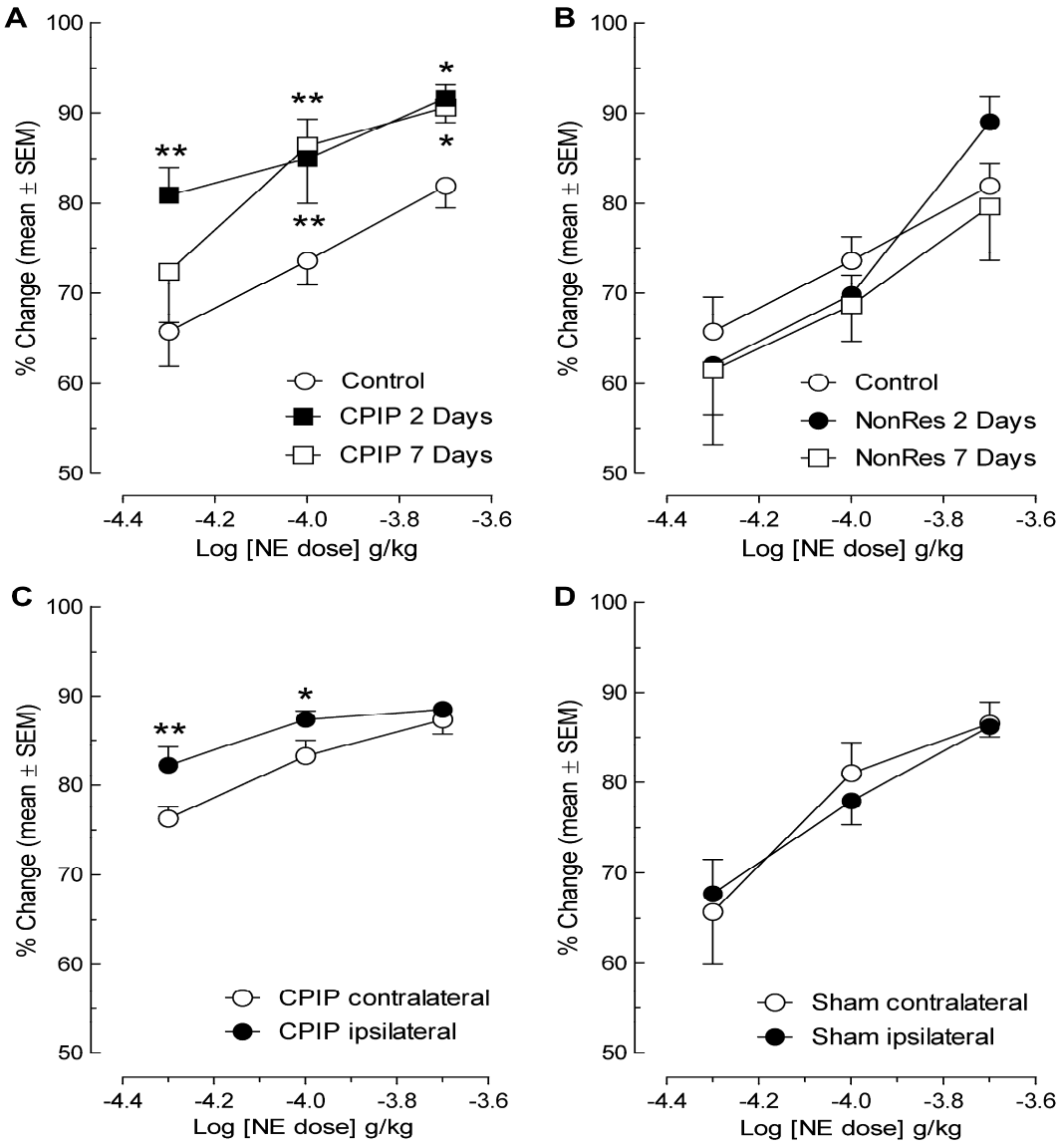

Figure 3. (**A**) Intra-arterial NE-evoked decreases in blood flow as determined by % change in the peak response to NE in CPIP responders. CPIP responders show enhanced NE-evoked decreases in blood flow for the 50, 100 and 200 μg/kg NE doses at both 2 and 7 days post-reperfusion ($^{*}P < 0.05$, $^{**}P < 0.01$). (**B**) Intra-arterial NE-evoked decreases in blood flow in CPIP non-responders. CPIP non-responders do not show enhanced NE-evoked decreases in blood flow for any dose of NE at either 2 or 7 days post-reperfusion. (**C**) I.v. NE-evoked decreases in blood flow in CPIP responders. CPIP responders show enhanced NE-evoked decreases in blood flow in the ipsilateral hind paw as compared to the contralateral paw for the 50 and 100 μg/kg NE doses at 2 days post-reperfusion ($^{*}P < 0.05$, $^{**}P < 0.01$). (**D**) I.v. NE-evoked decreases in sham control rats. There is no difference in NE-evoked decreases in blood flow between the ipsilateral hind paw as compared to the contralateral paw for any of the NE doses at 2 days post-reperfusion.

4.4.4 Effects of adrenergic antagonists on NE-evoked nociception and blood flow decreases in CPIP rats

Fig. 4 demonstrates the effects of the α_1-adrenergic receptor antagonist, prazosin, and the α_2-adrenergic receptor antagonist, yohimbine, on nociceptive behaviours when co-administered intradermally with 250 ng NE at 2 days post-reperfusion. One-way ANOVA reveals a significant main effect of group ($F_{6, 41} = 3.99$, $P < 0.01$). Post hoc analyses revealed that co-administration of prazosin at 2 and 10 μg ($^{*}P < 0.05$; $^{**}P < 0.01$), but not 0.4 μg, significantly reduced NE-evoked nociceptive behaviours. Co-administration of 2 and 10 μg ($^{*}P < 0.05$; $^{**}P < 0.01$), but not 0.4 μg, of yohimbine also significantly inhibited NE-evoked nociceptive behaviours.

Fig. 5 shows the effects of 10 μg doses of intra-arterial prazosin and yohimbine on close arterial NE-evoked decreases in blood flow. Two-way repeated measures ANOVA revealed significant main effects of group ($F_{2, 71} = 21.65$, $P < 0.0001$) and dose ($F_{2, 71} = 21.50$, $P < 0.0001$), as well as a significant group × dose interaction ($F_{4, 71} = 3.33$, $P < 0.05$). Post hoc analyses revealed that both prazosin pretreatment significantly reduced the peak decrease in flux at all NE doses ($P < 0.01$), while yohimbine pretreatment significantly reduced the peak decrease in flux at the 50 μg/kg NE dose ($P < 0.01$).

4.4.5 Effect of co-administration of sodium nitroprusside SNP or SIN-1 pretreatment on NE-evoked nociception in CPIP rats

To further verify whether vasoconstriction may be a mechanism for NE-evoked pain, we tested the effect of intradermal co-administration of NE with SNP, an NO donor and vasodilator, on NE-induced nociceptive behaviours at 2 days post-reperfusion in CPIP responders (Fig. 6A). One-way ANOVA revealed a significant main effect of group ($F_{3, 23} = 3.18$, $P < 0.05$). Post hoc analyses revealed a significant inhibition of NE-evoked nociception by the 100 and 500 μg doses of SNP ($P < 0.05$). We also tested the effect of pretreatment (30 min prior) with an anti-allodynic systemic dose (10 mg/kg) of the NO donor, SIN-1, on intradermal NE-induced nociceptive behaviours at 2 days post-reperfusion (Fig. 6B). One-way ANOVA revealed a significant main effect of group ($F_{2,21} = 6.15$,

$P < 0.01$). Vehicle pretreatment followed by 250 ng intradermal NE-induced significant nociceptive behaviours ($P < 0.01$). This effect was attenuated by pretreatment with 10 mg/kg SIN-1 ($P < 0.05$).

4.4.6 Effect of intradermal administration of a non-adrenergic vasoconstrictor or an eNOS inhibitor in CPIP rats

In a similar fashion as the NE experiments, we examined whether nociceptive behaviours could be induced in 2- and 7-day CPIP responders with an intradermal injection of the non-adrenergic vasoconstrictor, AVP, or an eNOS inhibitor, L-NIO). Fig. 7A shows the effect of intradermal injection of 500 ng AVP at inducing nociceptive behaviours in CPIP responders as compared to AVP in sham control rats and VEH injection in CPIP responders at 2 and 7 days post-reperfusion. Two-way ANOVA reveals significant main effects of treatment ($F_{1, 44} = 12.48$, $P < 0.01$) and condition ($F_{2, 44} = 4.38$, $P < 0.01$), but a non-significant treatment × condition interaction. Vehicle injection in CPIP rats did not produce significant nociceptive behaviours at 2 or 7 days post-reperfusion as compared to VEH injections in sham rats. AVP (500 ng) induced significant nociceptive behaviours at 2 and 7 days post-reperfusion, as compared to VEH in CPIP responders ($^{*}P < 0.05$, $^{**}P < 0.01$). AVP did not produce greater nociceptive behaviours in sham controls as compared to VEH injection.

Fig. 7B shows the effect of intradermal injection of 250 µg L-NIO at inducing nociceptive behaviours in CPIP-responders as compared to L-NIO in sham controls or VEH in CPIP responders at 2 and 7 days post-reperfusion. Two-way ANOVA reveals significant main effects of treatment ($F_{1, 45} = 16.93$, $P < 0.01$) and condition ($F_{2, 45} = 5.40$, $P < 0.01$), as well as a significant treatment × condition interaction ($F_{2, 45} = 4.25$, $P < 0.05$). Vehicle injection in CPIP rats did not produce significant nociceptive behaviours at 2 or 7 days post-reperfusion as compared to VEH injections in sham control rats. L-NIO-induced significant nociceptive behaviour at 2 days post-reperfusion as compared to VEH injections in CPIP rats ($P < 0.01$), but not at 7 days post-reperfusion as compared to VEH-injected CPIP rats. L-NIO also did not produce greater nociceptive behaviours in sham controls, as compared to VEH injection.

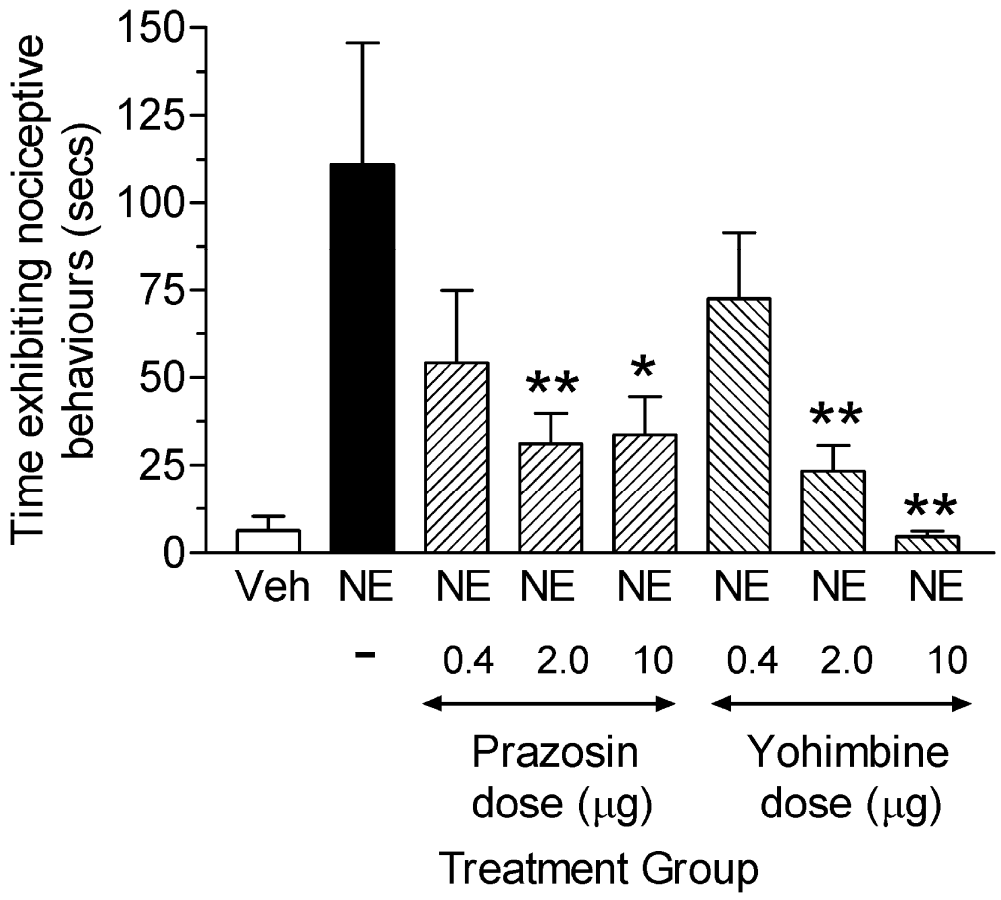

Figure 4. Effect of intradermal co-administration of α-adrenergic receptor antagonists on NE-induced nociception in CPIP rats. Co-administration of 2 and 10 μg of either prazosin or yohimbine significantly attenuates intradermal NE-evoked nociceptive behaviours in CPIP responders ($^{*}P < 0.05$, $^{**}P < 0.01$).

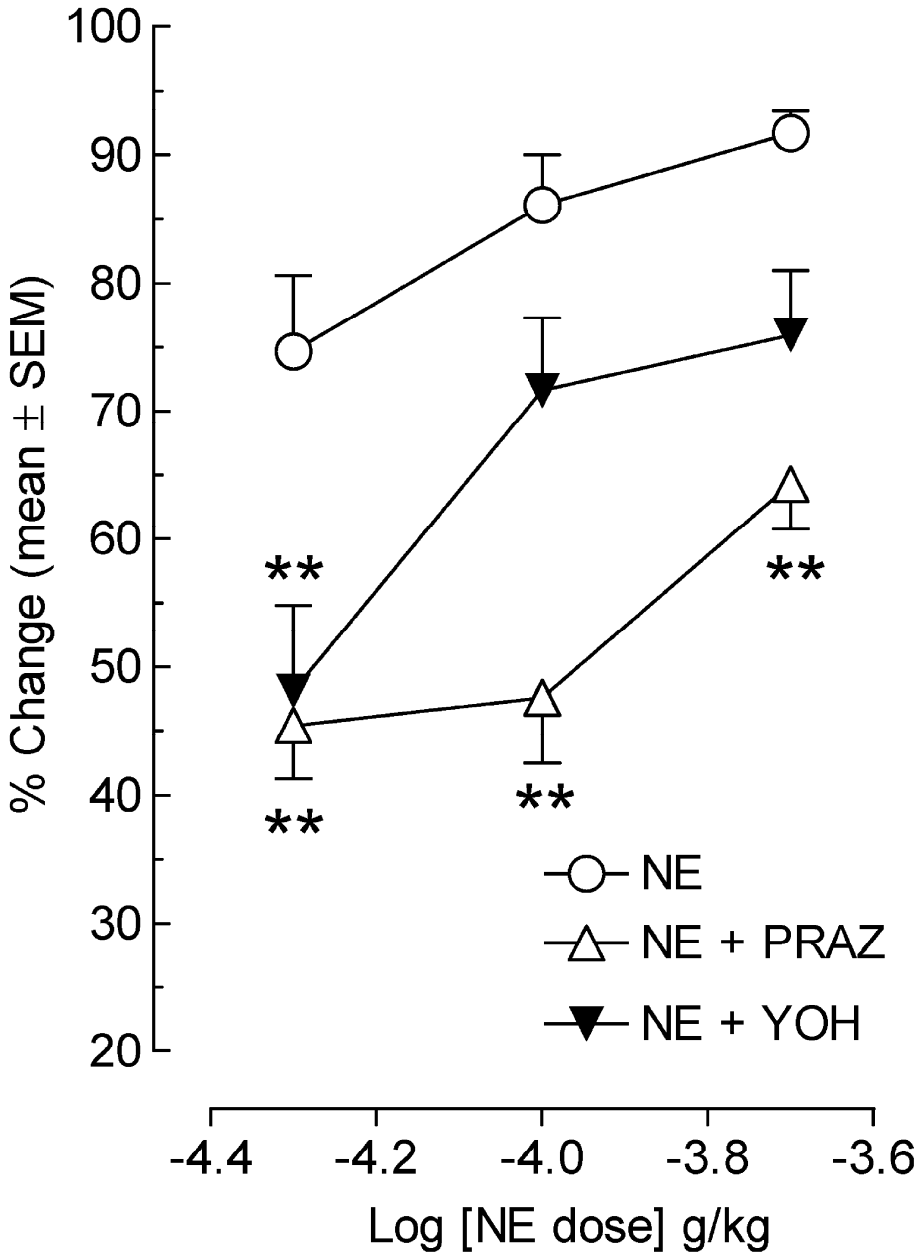

Figure 5. Effect of intra-arterial pretreatment with α-adrenergic receptor antagonists on intra-arterial NE-induced decreases in blood flow in CPIP responders. Administration of 10 μg prazosin results in an attenuated decrease in blood flow in response to intra-arterial NE at the 50, 100 and 200 μg/kg NE doses. Administration of 10 μg yohimbine results in attenuated decreases in blood flow in response to intra-arterial NE at the 50 μg/kg NE dose only. ($^{**}P < 0.01$).

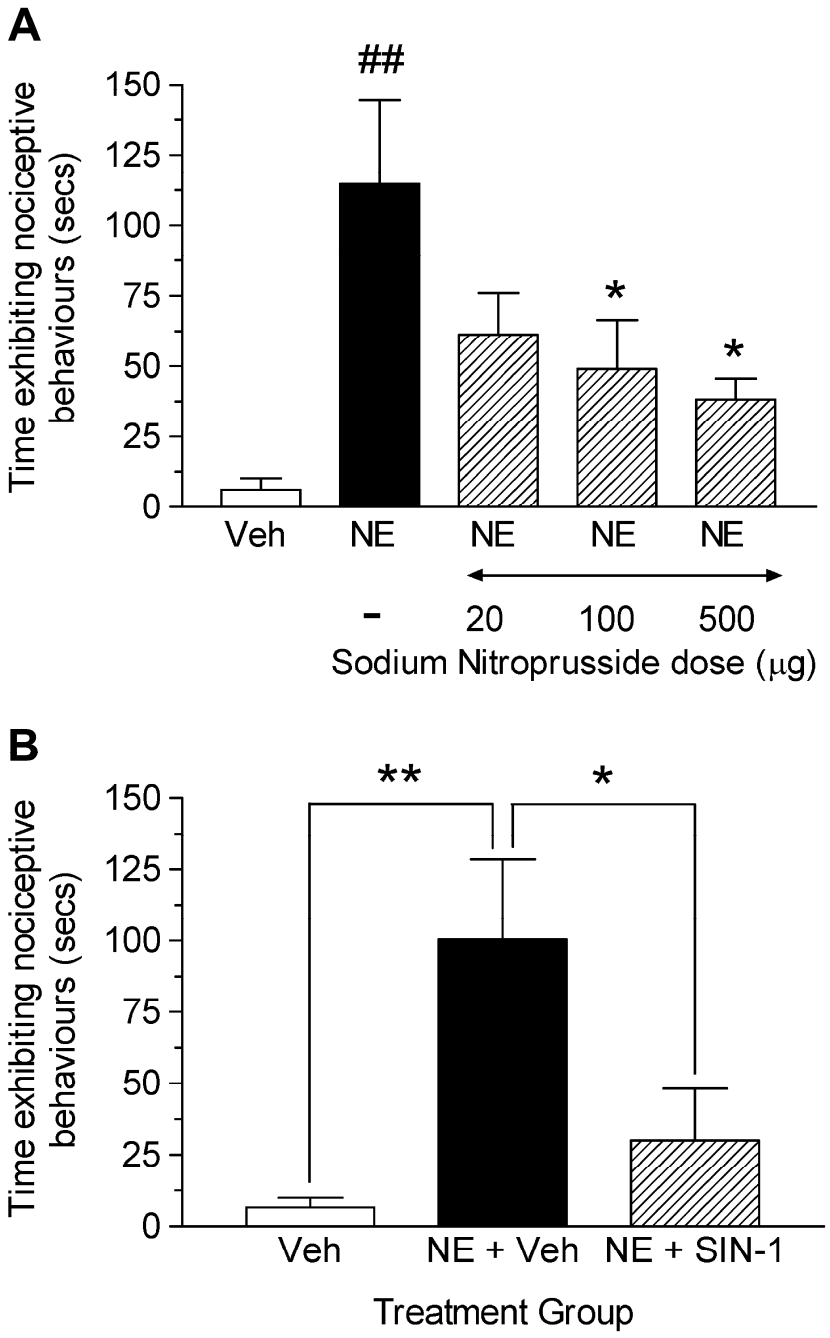

Figure 6. (**A**) Effect of intradermal SNP on intradermal NE-induced nociception in CPIP rats. NE alone again produced significantly greater nociceptive behaviours than VEH ($^{\#\#}P < 0.01$). Co-administration of 100 or 500 μg doses of SNP with 250 ng intradermal NE results in attenuated nociceptive behaviours as compared to NE alone ($^{*}P < 0.05$). (**B**) Effect of systemic pretreatment with SIN-1 on intradermal NE-induced nociception in CPIP rats. Administration of 10 mg/kg of SIN-1 30 min prior to 250 ng intradermal NE results in attenuated nociceptive behaviours as compared to NE alone ($^{*}P < 0.05$), which was significantly greater than VEH injection ($^{**}P < 0.01$).

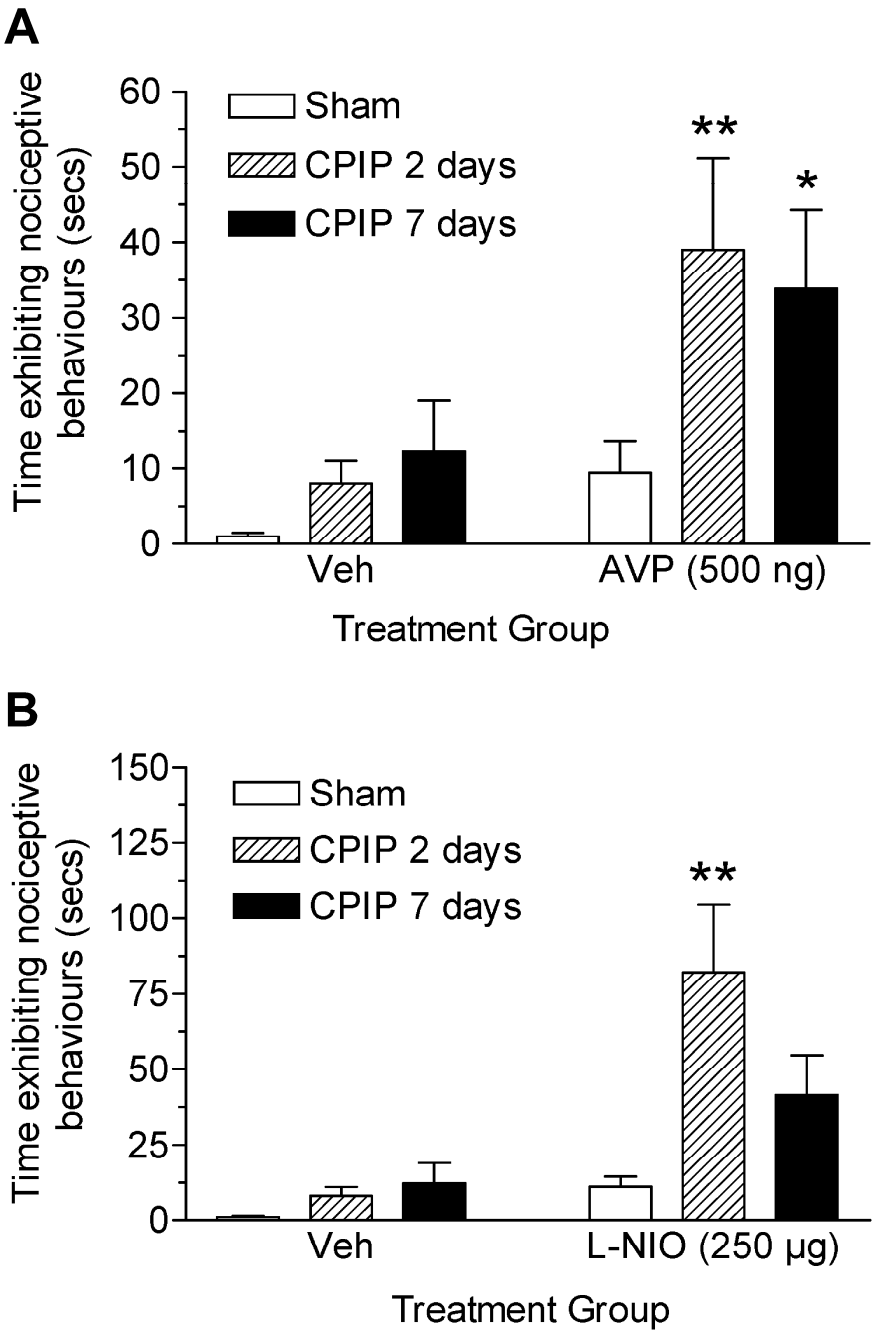

Figure 7. (**A**) Nociceptive effects of a non-adrenergic vasoconstrictor, vasopressin. Intradermal administration of 500 ng [Arg^8] vasopressin evokes significant nociceptive behaviours in CPIP rats as compared to VEH at both 2 and 7 days post-reperfusion ($^{*}P < 0.05$; $^{**}P < 0.01$). (**B**) Nociceptive effects of an eNOS inhibitor, L-NIO. Intradermal administration of 250 µg L-NIO evokes significant nociceptive behaviours in CPIP rats as compared to VEH at 2 days, but not at 7 days post-reperfusion ($^{*}P < 0.05$ and $^{**}P < 0.01$).

4.5 Discussion

Our main finding here is that CPIP rats with mechanical allodynia display both exaggerated NE-evoked nociceptive behaviours and enhanced vasoconstrictive responses to NE. CPIP responders displayed pain responses to intradermal NE, while sham rats did not. Our results parallel those found in CRPS patients where intradermal NE evokes intense abnormal pain lasting tens of minutes (Torebjörk et al., 1995; Ali et al., 2000). CPIP responders also show exaggerated decreases in blood flow in response to NE. CRPS patients also have increased vasoconstrictive responses to NE (Arnold et al., 1993; Birklein et al., 1997; Teasell & Arnold, 2004). Although early in the disease CRPS patients have reduced sympathetic function (Birklein et al., 1998a; Schürmann et al., 1999; Wasner et al., 1999; Schürmann et al., 2000; Wasner et al., 2001b), later in the disease CRPS patients have greater decreases in blood flow in response to sympathetic stimulation (Drummond et al., 2001; Wasner et al., 2001b; Baron et al., 2002; Ackerman et al., 2005), and exhibit denervation supersensitivity of vasoconstrictive adrenoceptors (Kurvers et al., 1995).

As well as exhibiting alterations relative to sham treatment after close arterial NE injections, the ipsilateral CPIP hind paw also showed vasoconstrictive hypersensitivity as compared to the contralateral paw following i.v. NE administration. Thus, while our relatively high doses of NE may have general systemic effects following close arterial administration, the vasoconstrictive hypersensitivity clearly depends on local alterations in the CPIP hind paw.

CPIP non-responders did not exhibit enhanced nociceptive responses to NE on day 2, but did on day 7. Thus, CPIP non-responders may have borderline pathology that worsens with time. Importantly, CPIP non-responders also did not exhibit significantly enhanced vasoconstrictive responses to NE. These findings strengthen the relationship between enhanced NE-induced nociceptive and vasoconstrictive responses in CPIP rats, by showing that rats with allodynia exhibit both painful and vascular NE hypersensitivity, while those without allodynia do not. The relationship is not absolute, since 7-day CPIP non-responders exhibited nociceptive responses to NE, but failed to exhibit

significantly greater NE-induced vasoconstriction. Of course, it is impossible to directly compare these phenomena, since we used different routes of administration, and the rats were conscious during behavioural experiments and anesthetised during laser Doppler experiments.

The relationship between NE-induced exaggerated nociception and enhanced vasoconstriction is further supported by the finding that both were significantly reduced by α_1- and α_2-adrenergic receptor antagonists. Mechanical allodynia in CPIP rats is also reduced by a non-selective adrenergic receptor antagonist, phentolamine, and an α_1-adrenergic receptor antagonist, prazosin (Xanthos & Coderre, 2008). While no clinical studies have examined whether α-adrenergic antagonists alleviate exaggerated NE-induced pain in CRPS patients, the α_1-adrenergic agonist, phenylephrine, produces painful responses similar to NE (Davis et al., 1991; Mailis-Gagnon & Bennett, 2004). In contrast, α_2-adrenergic antagonists, such as phentolamine (Arnér, 1991; Raja et al., 1991) and phenoxybenzamine (Ghostine et al., 1984; Muizelaar et al., 1997), are used to treat pain and allodynia in CRPS patients, and to diagnose SMP.

We also demonstrated that co-administration of a NO donor (SNP) results in a dose-dependent attenuation of NE-evoked nociception. SNP has strong vasodilator activity that occurs following the release of NO. We hypothesize that the NO-mediated vasodilatation counteracts the vasoconstrictive effect of NE, as has previously been documented (Thomas & Victor, 1998). SNP has also been shown to reduce thermal hyperalgesia induced by NE in capsaicin-treated skin in human subjects (Drummond, 1999), and we have shown that systemic SNP reduces mechanical allodynia in CPIP rats (Xanthos & Coderre, 2005). Administering another NO donor, SIN-1, at doses that are anti-allodynic, also attenuates NE-evoked nociception. NO-based vasodilators have been used to alleviate CRPS pain (Hyland, 1989; Manahan et al., 1993).

To further examine the relationship between vasoconstriction and NE-induced nociception, we tested the nociceptive effects of hind paw injection of a non-adrenergic vasoconstrictive agent, vasopressin, and an eNOS inhibitor, L-NIO. We found that both were able to induce nociception in CPIP rats. Although

an analgesic role for systemically and spinally administered vasopressin is known (Berkowitz & Sherman, 1982; Thurston et al., 1992), the effects of vasopressin on nociceptors have not been studied. However, after SNL, vasopressin evokes an excitation of axotomized afferents similar to that evoked by NE, suggesting vasoconstriction may be relevant to the effects of NE (Häbler et al., 2000). Also, both NE and vasopressin produce thermal hyperalgesia in capsaicin-treated skin in human subjects at doses that reduce blood flow (Drummond, 1998).

L-NIO, by inhibiting the synthesis of NO, reduces NO-induced vasodilatation. Although it produces no significant nociceptive behaviours in sham rats, L-NIO produces nociceptive behaviours in CPIP rats similar to those produced by NE and vasopressin. Thus, nociceptive behaviours are induced in CPIP rats either by enhancing vasoconstriction with adrenergic (NE) or non-adrenergic (vasopressin) vasoconstrictors, or by reducing NO-mediated vasodilatation with an eNOS inhibitor (L-NIO). It would be interesting to investigate parallels between vascular and nociceptive effects of vasopressin and NO in a future study. Although there is a large literature indicating that eNOS and NO are critical players in vasoregulation, and that NO is a potent vasodilator (Moncada et al., 1988), the role of eNOS and vascular NO in CRPS has received little attention. NOS is usually thought to be pro-nociceptive spinally (Tao et al., 2004), and intrathecal as well as systemic eNOS inhibitors attenuate pain in various pain models (Osborne & Coderre, 1999; Doursout et al., 2003; Naik et al., 2006b). In neuropathic pain, NO is often thought to play a pro-nociceptive role by generating nitrogen free radicals and causing vasodilatation that facilitates inflammatory processes (Ialenti et al., 1992; Levy et al., 2000; Levy & Zochodne, 2004). We hypothesize that in SMP, chronic tissue ischemia and vasoconstrictor hypersensitivity are over-riding factors that allow the major vasodilator role of endothelial NO to be beneficial rather than pro-nociceptive. Importantly, NOS activity and NO-dependent vasodilatation have been shown to be compromised after I–R injury (Wang et al., 1997), perhaps explaining why endogenous NO may not compensate for vasoconstrictor hypersensitivity in CPIP rats or CRPS patients.

In CRPS it has been suggested there is direct sympathetic–sensory coupling that underlies *de novo* adrenergic sensitivity in primary afferent neurons. Nerve injury induces sprouting of sympathetic fibers around the cell bodies of DRG neurons (McLachlan et al., 1993; Chung et al., 1996) and in the skin (Yen et al., 2006), as well as an upregulation of α-adrenoceptors in DRG neurons (Cho et al., 1997; Birder & Perl, 1999; Xie et al., 2001a). An upregulation of adrenergic receptors has been documented in the skin of CRPS-I patients (Drummond et al., 1996), although it has not been determined whether the receptors are on nociceptors. After SNL the number of axotomized L5 afferent nerves that respond to NE or sympathetic stimulation is dramatically increased following sustained vasoconstriction of the DRG, and the DRGs exhibit abnormal neuronal responses to the vasoconstrictors NE, angiotensin and vasopressin (Häbler et al., 2000), suggesting that sympathetic–sensory coupling is indirect and depends on intervening vasoconstriction.

Vasoconstriction-dependent *indirect* sympathetic–sensory coupling implies that vascular adrenergic hyper-responsiveness may be an alternative cause of adrenergic sensitivity of primary afferents in SMP. While not well studied in animal models of SMP, vascular adrenergic hyper-responsiveness has been demonstrated in various animal models of I–R injury. I–R injury of the rat tail (Seasholtz et al., 2001) and cremaster muscle (Lee et al., 1995) results in enhanced vasoconstrictor responses to adrenergic stimulation. In humans, chronic limb ischemia associated with peripheral arterial disease has been shown to result in enhanced responsiveness of α-adrenergic receptors (Jarajapu et al., 2001a). Indirect sympathetic–sensory coupling may depend more on upregulation of adrenoceptors on vascular smooth muscle cells than on primary afferents. Evidence shows there is an upregulation of both α_1- and α_2-adrenoceptors in vascular smooth muscle cells after I–R injury of the rat hind limb (Sapienza et al., 1996). The vascular α-adrenoceptor upregulation occurs within 1 h following reperfusion, much earlier than the onset of 7–14 days observed for upregulation in DRG cells after SNL or partial SNT (Cho et al., 1997; Birder & Perl, 1999). This is important since we have observed mechanical allodynia as early as 8 h after

reperfusion and abnormal NE-induced nociception at 2 days following reperfusion in CPIP rats. Although vascular α-adrenoceptor upregulation may contribute to the pathology in CPIP rats, it may be that more effective G-protein coupling of adrenergic receptors (Seasholtz et al., 2001), or reduced NE clearance after ischemia, may also be important factors in the NE hypersensitivity of CPIP animals.

4.6 Conclusions

We have shown here that exaggerated NE-induced nociception in CPIP rats is paralleled by enhanced vasoconstriction in the hind paw, and that both are relieved by α-adrenergic antagonists. NE-induced nociception is also relieved by a vasodilator and mimicked by a non-adrenergic vasoconstrictor or an eNOS inhibitor. We hypothesize that CPIP rats, and at least some CRPS-I patients, have an upregulation of vascular adrenergic receptors. The pain of CPIP and CRPS-I may depend on chronic tissue ischemia that is dependent on, or exacerbated by, an indirect sympathetic–afferent coupling with an intervening role of enhanced α-adrenoceptor-mediated vasoconstriction.

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Chapter 5

5. General Discussion

Furthering the understanding of the mechanisms of chronic pain via basic research is a vital part of improving currently available therapies and approaches to treatment. In addition to characterizing mechanisms that contribute to sensory abnormalities in widely used animal models of chronic pain, it is often important to reframe the critical mechanistic questions with original approaches to basic research and new perspectives on clinical conditions. This can particularly benefit those clinical conditions that are poorly understood. It can also serve to expand characterizations of basic mechanisms in new directions. This thesis describes the development of a novel animal model of chronic pain produced by hind paw I-R injury, which is proposed to be particularly relevant to CRPS-I. Systematic study of nociception after hind paw I-R injury is an approach that has not been used before experimentally in an animal model. Abnormal interaction of the SNS and nociceptive systems, which is described in some CRPS patients and those with neuropathic pain, is theorized to be particularly relevant to this animal model and our results suggest that it may contribute to pain.

5.1 Contributions of ischemia to CPIP and CRPS, and a comparison of animal models of CRPS-I

Chapter 2 describes a novel animal model developed with the use of a 3 hour tourniquet ischemia of the rat hind paw, and termed CPIP. The ischemia induces 100% occlusion of blood flow (see Appendix A). With reperfusion, the ipsilateral hind paw shows prominent hyperaemia as measured by increased blood flow (Appendix A) and skin temperature (Chapter 2) that persist for at least two hours. Oedema as measured by plasma extravasation lasts for up to 12 hours post-reperfusion. Light microscopy does not reveal evidence of nerve injury underneath the tourniquet, and conduction velocity in the sural nerve is normal (Laferrière et al., 2007). Robust mechanical allodynia, cold allodynia, and mechanical hyperalgesia are measured at 8 hours and persist for at least 4 weeks in 70% of the animals. Contralateral symptoms and spontaneous pain behaviours

are also detected. A pharmacological trial with an antioxidant and a free radical scavenger, NAC and TEMPOL, reversed mechanical allodynia in CPIP rats (Chapter 2).

CPIP is one of a handful of animal models for CRPS-I, and the first systematic study that examines nociception after a hind paw I-R injury. It is theorized to be particularly relevant to both signs and symptoms of CRPS-I, for which pain, inflammatory signs, vascular abnormalities, and ischemia are particularly prominent, as well as no major nerve injury as confirmed by no alterations in nerve conduction velocity. A variety of evidence has accumulated in the recent years suggesting that skin and muscle tissue are ischemic in some CRPS patients. Low skin capillary oxygenation (Koban et al., 2003), increased skin lactate (Birklein et al., 2000c), impaired nutritive skin blood flow (Kurvers et al., 1995), oxidative stress (van der Laan et al., 1998b), arteriovenous shunting (Matsumura et al., 1996; Schürrman et al., 2001), impairment of high-energy phosphate metabolism (Heerschap et al., 1993), increased endothelin-1, and decreased NO (Groeneweg et al., 2006) have all been found in the limbs of CRPS-I patients. There are also studies showing successful treatment of CRPS with free radical scavengers (Goris, 1985; Goris et al., 1987; Zuurmond et al., 1996; Perez et al., 2003) and reports that ischemia during surgery can sometimes result in prolonged post-operative pain and CRPS (Sherman et al., 1986; Callum & Bradbury, 2000; Berga et al., 2002).

Preliminary evidence from our laboratory suggests that CPIP hind paw muscles at 2 days and 7 days post-reperfusion show decreased capillary density, impaired mitochondrial activity, upregulation of cytokines, NFκB, and lipid peroxidation, as well as other evidence of ongoing ischemia such as increased lactate levels and increased pain with exercise (Laferrière et al., 2007, 2008). A drop of pH in experimental ischemia with a tourniquet in human volunteers is correlated with pain during muscular activity (Lewis et al., 1931; Issberner et al., 1996) suggesting chronic ischemia after reperfusion is likely to result in acidosis that underlies chronic muscular pain. Acute inflammatory mechanisms after I-R injury also interact with tissue acidosis caused by ischemic states, and may serve

to exacerbate pain (Reeh & Steen, 1996). Consistent with consequences of I-R injury described in the literature, CPIP rats display a reperfusion-induced inflammatory-like response demonstrated by a pattern of initial hyperaemia and oedema (Chapter 2), including upregulation of cytokines (Laferrière et al., 2007). This is followed by a chronic neuropathic-like pain phase involving nociceptive abnormalities with "cold" and ischemic limbs as evidenced by the pallor of the muscles of the plantar foot and decreased capillary perfusion, detectable up to 21 days post-reperfusion (unpublished observations; Laferrière et al., 2007). These results are consistent with the theories of neurogenic inflammation in the pathogenesis of CRPS-I, in which an early exaggerated inflammatory response is thought to occur in response to an initial injury, which subsequently leads to chronic symptoms (Oyen et al., 1993; Birklein et al., 2001; Weber et al., 2001; Birklein, 2005).

Painful limbs of both chronic CRPS-I and CRPS-II patients exhibit similar temperature and blood flow abnormalities. Hence, CRPS patients can present with "hot" symptoms (increased skin temperature, oedema, sweating), "cold" symptoms (decreased skin temperature, cyanosis, dryness), and mixed "hot" and "cold" symptoms, which can vary with patient and/or disease progression. Clinical studies usually describe "hot" symptoms as occurring primarily early in the syndrome (months), and "cold" symptoms as occurring later in chronic CRPS patients (years) (Bonica, 1990; Veldman et al., 1993; but see Bruehl et al., 2002). Like CPIP, animal models using a nerve injury, particularly CCI, also show important temperature and blood flow abnormalities which tend to progress from an initial "hot" phase to a later "cold" phase (Bennett & Ochoa, 1991; Wakisaka et al., 1991). Nerve injury pain is clearly more relevant to CRPS-type II, for which a major nerve injury is detected and often initiates pain complaints.

Ludwig et al. (2007) recently assessed the effects of a 3 hour occlusion of the femoral artery on nociceptive response in Wistar rats. A sham group was subjected to anesthesia and surgery to expose and free the artery, but did not undergo vascular occlusion. Interestingly, both groups developed short-lasting sensory abnormalities making the authors conclude that I-R injury by arterial

occlusion does not induce long-term nociceptive symptoms. While this study was the first to attempt to replicate our results, their conclusions can be criticized for several reasons. First, unlike CPIP, their ischemia produced by arterial occlusion was not complete, as laser Doppler flux levels were only reduced by approximately 65 percent. Second, again unlike in our studies with CPIP rats, they did not observe any reactive hyperaemia as would be expected with such a prolonged ischemia. Third, systemic heparin was used throughout the ischemic period to prevent thrombosis of the femoral artery. It is well known that heparin can attenuate consequences of I-R injury such as oedema (Druid & Rammer, 1989), skeletal muscle injury (Wright et al., 1988), and endothelial dysfunction (Sternbergh et al., 1993). Fourth, sham rats exhibited similar sensory abnormalities, suggesting that the surgery may have caused injury to the adjacent femoral nerve. Therefore, for these reasons, we believe it cannot be concluded as suggested by Ludwig et al. (2007) that I-R injury does not induce sensory abnormalities, or that it cannot be used to produce an animal model of CRPS-I.

More recently, Schoen et al. (2007) studied the nociceptive and inflammatory effects of 3-hour tourniquet ischemia, although the tourniquet was placed in the rat thigh above the trochanter major. Here, like our method, laser Doppler flux measurements during the tourniquet placement indicated complete ischemia. With release of the tourniquet, prominent reactive hyperaemia was noted, as we found, but unlike the Ludwig et al. (2007) study. The authors observed persistent tissue ischemia and inflammation indicated by a significant reduction in functional capillary density, and increased leukocyte rolling and adherence to the endothelial lining at the postcapillary venules in hind limb muscle. Most importantly, rats exhibited significant hind paw oedema and mechanical allodynia, but not heat or cold hyperalgesia, at 24 hours post-reperfusion. These results resemble those in CPIP rats in which there is also persistent reactive hyperaemia, reduction of capillary density, upregulation of cytokines, and swelling of endothelial cells for at least 2 days post-reperfusion (Laferrière et al., 2007; Appendix A). Unfortunately, the study by Schoen et al (2007) did not follow the animals past 24 hours. Unlike the CPIP rats (Laferrière

et al., 2007), rats with the higher thigh tourniquet exhibited a reduction of nerve conduction velocity, as well as mitochondrial swelling, and peri- and endoneurial oedema in the sciatic nerve. These signs are all indicative of major nerve injury early after tourniquet application, and hence may make this severe type of I-R injury more similar to CRPS-II. As described in chapter 1, animal models that are said to mimic CRPS-I have used techniques such as electrical nerve stimulation (Vatine et al., 1998), nerve needle stick (Siegel et al., 2007), tibial fracture (Guo et al., 2004), and intra-arterial infusions of various substances such as a free radical donor (Van der Laan et al., 1997a), substance P (Gradl et al., 2007), or extracts from inflamed muscle with soft tissue trauma (Gradl et al., 2006), which avoid producing major nerve injury.

A couple of studies have focused on the role of free radical and neurogenic inflammation on the pathogenesis of CRPS-I. A 24 hour free radical donor infusion was suggested as an animal model of CRPS-I with rats exhibiting sensory abnormalities up to 4 weeks (Van der Laan et al., 1997b; Van der Laan et al., 1998a). Several signs of inflammation such as oedema and hyperaemia were noted. Mechanical allodynia and heat hyperalgesia persisted for up to 4 weeks. Spontaneous pain behaviours and contralateral pain behaviours were recorded for about 1 week. Muscle fiber injury was also noted. Studies with this animal model come to similar mechanistic conclusions as our studies with CPIP rats, since free radical generation is a major consequence of prolonged ischemia, and CPIP symptoms are reduced by free radical scavengers and antioxidants. They are also consistent with clinical studies showing free radical damage in CRPS (Van der Laan et al., 1998b), and several studies showing analgesic effectiveness of free-radical scavengers or antioxidants in CRPS (Goris et al., 1987; Oyen et al., 1993; Perez et al., 2003). Unfortunately, no studies have followed up the original model description. More recently, infusion of substance P for 24 hours (Gradl et al., 2007) was also proposed as to mimic CRPS-I, since substance P is released with neurogenic inflammation in these patients (Weber et al., 2001). Inflammatory changes such as increased leukocyte rolling and increased endothelial-leukocyte interactions were shown, although no ischemia. Apoptosis of muscle tissue was

also not observed. Hence, both infusion methods of free radicals and substance P may be consistent with a mechanism whereby neurogenic inflammation contributes to the "hot" phase of CRPS-I and CPIP. However, they may not relate to the chronic ischemic changes found in CRPS-I. On the other hand, the increased leukocyte rolling after substance P infusion suggests there may be endothelial cell injury, and the muscle injury after free radical donor infusion may have been caused by persistent ischemia associated with free radical-induced vascular injury, but this was not specifically measured.

Guo et al. (2004) recently developed an animal model of CRPS-I produced by fracture and casting of the rat tibia (Guo et al., 2004). This produces long-lasting mechanical allodynia for up to 16 weeks. Recent studies indicate cytokine upregulation, osteopenia, and central sensitization after fracture and casting, and that anti-NGF, glucorticoids, and anti-inflammatory drugs reduce sensory symptoms in these rats (Guo et al., 2006; Sabsovich et al., 2008a; Sabsovich et al., 2008b). This animal model displays great face validity, since fracture is one of the most common causes of CRPS-I (up to 44%; de Mos et al., 2007). However, this model may not generalize to CRPS-I patients that never had fractures, and it has not addressed that most patients with fracture and casts (over 97% by some estimates; see Dijskstra et al., 2003) do not go on to develop severe chronic pain and CRPS. Animals that received only casting also developed nociception for several weeks (Guo et al., 2004), suggesting complex interactions between tight casting, possibly due to ischemia, and fracture. It would be interesting for future studies to disentangle the individual mechanisms that lead to the syndrome, and how these may relate to CPIP. Recent studies have so far focused on inflammatory mechanisms and changes in bone after tibial fracture and casting that have been argued to be relevant to CRPS-I.

Gradl et al. (2005) recently showed that controlled-impact soft tissue trauma, or infusion of inflamed muscle tissue into uninjured rats, results in mechanical allodynia 24 hours later. Soft tissue trauma, but not infusion of inflamed muscle, also resulted in apoptotic muscle tissue, and evidence of tissue hypoxia, as demonstrated by decreased capillary perfusion and increased β-

nicotinamide adenine dinucleotide (NADH) autofluorescence. However, both methods induced a vascular inflammatory response as measured by increased leukocyte rolling and endothelial cell-leukocyte interaction. With the CCI model as a comparison animal model of CRPS-II, a follow-up study induced a combination of both soft tissue trauma and infusion of inflamed muscle in order to mimic CRPS-I (Gradl et al., 2006). This procedure resulted in mechanical allodynia for at least 4 days, as well as heat hyperalgesia and spontaneous pain behaviours for 24 hours. As expected, CCI rats showed more significant pain behaviours. However, in the CRPS-I model there was decreased capillary density and increased NADH autofluorescense, both persisting up to 14 days, and increased rolling leukocytes up to 4 days, all of which were not detectable in CCI rats. CRPS-I often involves soft tissue injury as after crush injury or sprains (de Mos et al., 2006), hence this model displays good face validity. The initial inflammatory response and the persisting ischemia processes also resemble our results with CPIP tourniquet ischemia (Laferrière et al., 2007) and the general pattern of CRPS-I patients, but not CCI, suggesting that ischemic mechanisms may be particularly important in injuries that do not directly involve major nerves, and that ischemic mechanisms can accompany pain, without the presence major nerve injury.

Our CPIP model focuses on the prominent ischemic processes detected in most CRPS-I patients, and the similarities of symptoms between CPIP rats and CRPS patients suggest that this approach can account for the variety of initiating injuries of CRPS-I, which may even be spontaneous and idiopathic (De Mos et al., 2007). The most widely used animal models of CRPS-II induce nociceptive symptoms that last several weeks to months, and the prolonged duration of sensory abnormalities are considered important for establishing the validity of these animal models. It is important that investigators establish the maximal duration of symptoms in the various animal models of CRPS-I, particularly those that have not been studied beyond two weeks. Also, for CPIP to become a useful research tool to model CRPS-I, it is important that it be further characterized and replicated by other research groups. To this day, CPIP has been replicated in

multiple studies from our laboratory, and CPIP-associated mechanical allodynia has been shown to extend past 4 weeks for at least 2 months (Millecamps et al., 2008; personal communications and unpublished data). CPIP has also been produced in mice (Swiss and C57/Bl6) (Millecamps et al., 2008) and other strains of rats (Sprague-Dawley and Wistar rats) (Xanthos DN; unpublished observation), all findings which strengthen the validity of this animal model.

5.2 Antisympathetic treatments in CPIP

Results presented in chapters 3 contribute further to the validation of CPIP as an animal model of CRPS-I. Consistent with the reports of positive responses to anti-sympathetic treatments in some CRPS-I patients (Price et al., 1998, Stanton-Hicks, 2003; Lake, 2004), mechanical allodynia in CPIP rats is partially relieved after treatment with guanethidine. Phentolamine and prazosin are also effective at reducing allodynia in CPIP rats, consistent with clinical studies demonstrating the utility of mixed α_1/α_2 adrenergic antagonists (Raja et al., 1991; Arnèr, 1991; Malik et al., 1998) and α_1-adrenergic antagonists (Abram & Lightfoot, 1981; Stevens et al., 1993) in CRPS patients. We also found that these drugs are more effective at 2 days post-reperfusion than 7 days, consistent with several clinical reports that anti-sympathetic treatments are more effective when used early in the syndrome (Bonica, 1990; AbuRahma et al., 1994; Singh et al., 2003; Ackerman & Zhang, 2006).

Pain that responds to anti-sympathetic procedures has been called SMP and has been primarily associated with CRPS-I due to the long use of anti-sympathetic treatments in CRPS-I patients. No animal models of CRPS-I have investigated the anti-allodynic effects of anti-sympathetic procedures. In chapter 3 studies, two treatments of 30 mg/kg guanethidine s.c. were found to provide significant long-lasting reduction of mechanical allodynia when administered early after reperfusion (2-3 days post). In animal models of CRPS-II, three studies reported anti-allodynia with guanethidine treatment in animals with nerve injury. A single dose of 30 mg/kg guanethidine reduced mechanical allodynia 10 days after PSNL in mice (Malmberg & Basbaum, 1998). The same dose of guanethidine reduced mechanical allodynia when administered several months

after PSNL in rats (Shir & Seltzer, 1991), and reduced mechanical allodynia two weeks after SNL in rats (Kim et al., 1993). Hence it appears that CPIP, at 2 days post-reperfusion, resembles PSNL and SNL, which both show reductions in mechanical allodynia with guanethidine treatment.

Phentolamine at doses of 5 mg/kg i.p. also significantly relieves mechanical allodynia at 2 days post-reperfusion, but not at 7 days post-reperfusion, in CPIP rats. This further suggests that CPIP rats display symptoms of SMP early post-reperfusion. Phentolamine's effectiveness in animals is particularly relevant to CRPS, since it has also been used as a diagnostic agent for SMP in CRPS patients (Arnér, 1991; Raja et al., 1991). Two studies found anti-allodynic effects with 4 and 5 mg/kg of phentolamine i.p. in SNL rats (Kim et al., 1993; Lee et al., 1999). However, other studies with 4 or 5 mg/kg phentolamine were unable to replicate this result in SNL rats (Ringkamp et al., 1999b; Park et al., 2000; Nam et al., 2001). Strain differences to phentolamine after SNL have been suggested to explain differences, since some rat strains are particularly sensitive to phentolamine, while others are insensitive (Lee et al., 1997). Interestingly, naive Long Evans and Sprague-Dawley have been shown to exhibit greater flinching behaviours than Wistar rats in response to intradermally co-administered NE and α-β-methylene-ATP (Waldron & Sawynok, 2004). The Long-Evans strain used in our studies, to our knowledge, has not been tested before for phentolamine nociception studies, and appears to show normal hypotension in response to phentolamine administration (Winn et al., 1986). In relation to chronic pain models, our results resemble those in SNL rats, but strain difference studies are warranted to clarify the generalizability of our findings.

The α_1-selective adrenergic antagonist prazosin, but not the α_2-selective adrenergic antagonist yohimbine, was more effective in reducing CPIP mechanical allodynia than phentolamine. Doses of 1-5 mg/kg i.p. prazosin significantly attenuated mechanical allodynia at 2 days post-reperfusion, while 2.5-5 mg/kg i.p. doses also relieved mechanical allodynia at 7 days post-reperfusion. Terazosin, which is more selective that prazosin, was found to relieve mechanical allodynia in SNL rats (Lee et al., 1999). However, s.c. prazosin was

ineffective in reducing mechanical hyperalgesia or heat hyperalgesia in PSNL rats (Tracey et al., 1995). Prazosin at doses of 0.5-2 mg/kg i.p. has also been shown to be effective against cold allodynia after tail nerve injury (Kim et al., 2005a; Kim et al., 2005b), and doses of 1-5 mg/kg i.p. reduce heat hyperalgesia in CCI rats (Hord et al., 2001). On the other hand, yohimbine at doses of 1-5 mg/kg i.p. was ineffective in CPIP rats, at either 2 days post-reperfusion or 7 days post-reperfusion. While these results are consistent with tail nerve injury for which yohimbine at 0.5-2 mg/kg i.p. was ineffective in reducing cold allodynia (Kim et al., 2005a; Kim et al., 2005b), they are even better supported by Lee et al. (1999) who found that idazoxan, rauwolscine and yohimbine were unable to relieve mechanical allodynia in SNL rats. However, our results contrast with anti-allodynia produced by the α_2-adrenergic antagonists yohimbine, atipamezole, and L659,066 after mouse tibial nerve transection (Kingery et al., 2000). They also contrast with studies showing that another α_2-adrenergic antagonist, SKF86466 reduces heat hyperalgesia in CCI rats (Hord et al., 2001), and s.c. yohimbine reduces both mechanical and heat hyperalgesia in PSNL rats (Tracey et al., 1995).

Overall, our results most closely resemble studies in SNL rats. As discussed, these studies have reported effectiveness of guanethidine, phentolamine and selective α_1-adrenergic antagonists, but not α_2-adrenergic antagonists, specifically against mechanical allodynia in SNL rats. SNL rats have often been reported to exhibit SMP since surgical sympathectomy and anaesthetic blocks of the lumbar sympathetic ganglia reduce mechanical allodynia, cold allodynia, and heat hyperalgesia at various time points after the nerve injury (Kim et al., 1993; Choi et al, 1994; Kinnman & Levine, 1995; Chung et al., 1996; Park et al., 2000). Therefore, it is reasonable to conclude that CPIP rats display evidence of SMP similar to the well characterized SNL animal model. The evidence for SMP is strongest at 2 days post-reperfusion, but is weaker at later time points. In our studies, we did not find a significant reduction of mechanical allodynia when guanethidine or phentolamine was administered 7 days post-reperfusion. Other drugs such as morphine, dexamethasone, and pregabalin are also more effective in reducing CPIP mechanical allodynia at 2 days post-

reperfusion rather than 7 days post-reperfusion (Millecamps & Coderre, 2008). Interestingly, pregabalin which has the highest effectiveness in CPIP rats is also reported to be very potent in SNL rats that respond to anti-sympathetic treatments. On the other hand, pregabalin is less effective in rats with a tibial/sural nerve transection that do not respond to anti-sympathetic treatments (Han et al., 2006; Han et al., 2007). All of the above results suggest that the mechanisms that maintain mechanical allodynia in CPIP rats may change over time, causing a reduction in the responsiveness to anti-sympathetic treatments.

Our time-course studies showed that guanethidine and effective adrenergic agents produce dose-dependent anti-allodynic effects that persist for at least an hour. Since vasodilatation follows successful sympathetic blockade, we hypothesized that this may be a relevant anti-nociceptive mechanism in CPIP by attenuating potential vasospasms and no-reflow, well known consequences of I-R injury. This is consistent with known hind limb vasodilatory effects of chemical sympathectomy or sympathetic blockade in rats (Clark & Phelan, 1975; Angus et al. 1978) and after human I-R injury (van Dielen et al., 1998; Povlsen & Sirsjo, 1999). Systemic administration of phentolamine and prazosin would also be expected to induce vasodilatation, and counteract I-R injury-induced ischemia by reducing the vasoconstrictive effects of NE on vascular smooth muscles. The further demonstration that a NO donor vasodilator also reduces mechanical allodynia adds further credence to the suggested vasodilatory mechanism of action of antisympathetic treatments. Since drugs were more effective at 2 days post-reperfusion, it is probable that vasodilatation is more effective against vasospasms that may be occurring early after I-R injury, but may be less effective for no-reflow which is a late phenomenon of I-R injury (Allen et al., 1995; Nanobashvili et al., 2003; Laferrière et al., 2007). Similarly, in CRPS, it is suggested that the contributions of the SNS to pain may change over time (Gradl & Schürmann, 2005; Schattschneider et al., 2006a) and anti-sympathetic treatments may be much more effective when initiated early in the syndrome (Aburahma et al., 1994; Schwartzmann et al., 1997; Singh et al., 2003 Ackerman

& Zhang, 2006). Hence, it appears that CPIP does resemble the overall picture seen in CRPS patients with SMP.

5.3 The role of the α_2-adrenergic receptor in CPIP

In chapter 3, it was shown that while systemic administrations of the mixed α_1-/α_2-adrenergic antagonist, an α_1-adrenergic antagonist, and an α_2-adrenergic agonist all reduce mechanical allodynia in CPIP rats, systemic administration of an α_2-adrenergic antagonist does not. However, in chapter 4, local injection of both the α_1-adrenergic antagonist and the α_2-adrenergic antagonist reduced NE-induced nociceptive behaviours and vasoconstrictive hypersensitivity in CPIP rats. Hence, it appears that either the route of administration (i.p. versus intradermal and close-arterial) or the action on the particular type of nociception (mechanical allodynia versus NE-induced nociceptive behaviours) may account for the ability of α_2-adrenergic receptor to produce anti-nociceptive effects in CPIP rats.

The action of a locally administered versus systemically administered α_2-adrenergic agent is likely to be different. Hence, while systemic administration will have effects on receptors on smooth muscle cells, it is also likely to directly influence the SNS. An action of α_2-adrenergic antagonists at SPGN is hypothesized because there is a large distribution of α_2-presynaptic receptors on these neurons. This would in turn result in increased NE release. Indeed, 1 mg/kg i.v. yohimbine results in significantly increased NE levels both in plasma systemic circulation and in various brain regions (Szemererdi et al., 1991). Increased NE spillover in the systemic and forearm circulation in human volunteers is also seen after i.v. administration (Grossman et al., 1991). The finding that systemic yohimbine had no anti-allodynic effect in CPIP rats, and the non-specific α-adrenergic antagonist phentolamine had less of an effect than prazosin are consistent will an action by which antagonism of α_2-autoreceptors enhance NE release, increase vasoconstriction, may therefore fail to produce anti-allodynia. On the other hand, systemic administration of clonidine reduces NE release. A ~0.2 mg i.v. infusion of clonidine in human volunteers results in central

SNS suppression and decreases plasma NE levels, including levels in the cerebral circulation (Lambert et al., 1998). In the rat, doses of 0.01 mg/kg i.v. clonidine result in significant decreases of plasma NE and also pressor responses induced by sympathetic stimulation (Szemererdi et al., 1988). In CPIP rats, it is consistent that systemic clonidine will decrease SPGN NE release, relieve vasospasms, reduce ischemia and therefore relieve allodynia.

CNS and direct hind paw effects of α_2-adrenergic agents are likely in our studies using i.p. administration. This is particularly true for spinal α_2-adrenoceptors, which are well known to be involved in nociception (Pertovaara, 2006). Thus, descending adrenergic pathways that terminate in the spinal cord dorsal horn have been implicated in the antinociceptive actions of other analgesic drugs such as morphine or gabapentin (Wigdor & Wilcox, 1987; Millan, 2002; Tanabe et al., 2005). Intrathecal clonidine is well known to reduce various pain symptoms after nerve injury (Puke et al., 1991; Pan et al., 1998). On the other hand, several studies have shown exacerbation of nociception by α_2-adrenergic antagonists: i.t. yohimbine in CCI (Satoh & Omote, 1996), i.t. and i.p. atipemazole in subgroups of rats that do not develop SNL (Xu et al., 1999) similar to "non-responders" in our studies), and i.p yohimbine in tail nerve injury for subgroups of rats that respond to phentolamine (similar to a clinical SMP classification) (Kim et al., 2005b). In CRPS-I, it has been shown that epidural clonidine infusion can provide significant pain relief (Rauck et al., 1993). Hence, the ability of i.p. clonidine, but not yohimbine, to provide anti-nociception in CPIP is also consistent with the spinal actions of these two drugs on α_2-adrenergic receptors.

In addition, antinociception after peripheral activation of α_2-adrenergic receptors has also been documented. Topical clonidine administration either via tail immersion or intradermal injection is anti-nociceptive in the tail-flick test (Dogrul & Uzbay, 2004; Dogrul et al., 2006). In addition, a clonidine cream dose-dependently relieves rat SNL thermal hyperalgesia and mechanical allodynia which is reversed by 10 mg/kg i.p. yohimbine (Li et al., 2007). Several studies have shown inhibitory effects of α_2-adrenergic receptor agonists on DRG

neuronal activity. Clonidine application reduces hyperpolarisation activated currents and the firing frequency of axotomized DRG neurons (Yagi & Sumino, 1998). Specifically clonidine, but not phenylephrine, has been shown to inhibit electrically-stimulated calcium responses in a much greater proportion of DRG cells in SNL rats than in normal rats (Eisenach et al., 2005). In CRPS-I, topical clonidine via patch also reduced pain in a small group of patients with SMP (Davis et al., 1991).

Delineating a direct pro-nociceptive or an anti-nociceptive role for peripheral α_2-adrenergic activation is complex, however, since activation of injured primary afferent fibers by NE has often been reported to be mediated via α_2-adrenergic receptors. Hence, yohimbine blocks NE-induced outward currents in axotomized DRG neurons (Abdulah & Smith, 1998) and NE effects on calcium currents in DRG neurons after CCI (Honma et al., 1999). Yohimbine also blocks sympathetically-evoked afferent activity in rats with SNL (Leem et al., 1997) or neuromas (Chen et al., 1996). NE-induced activation of C-fibers after rabbit ear nerve injury is also mediated by α_2-adrenoceptors (Sato & Perl, 1991), and α_2-adrenergic receptors increase in DRG neurons after PSNL (Birder & Perl, 1999). Interestingly, rekindling of mechanical allodynia in nerve-injured animals that underwent successful sympathectomy was elicited by cutaneous administration of both NE and an α_2-adrenoceptor agonist, and blocked by α_2-adrenergic receptor antagonists (Moon et al., 1999). Yohimbine also blocks NE-induced aggravation of mechanical hyperalgesia in PSNL (Tracey et al., 1995). These results support the interpretation that peripheral α_2-adrenoceptors mediate the NE-induced pain after nerve injury. As discussed in chapter 1, however, other studies have shown that only α_1-adrenergic receptor receptors are involved in NE-induced pain. For example, in monkey SNL, α_1-adrenergic receptor antagonists, but not α_2-adrenergic antagonists, block NE sensitivity in C-fibers (Ali et al., 1999). It should be highlighted that some of these potentially contradictory findings could be explained by species or strain differences in the responsiveness to adrenergic antagonists (Lee et 1997; Banik et al., 2001). Specificity of drug used related to dose may also affect the results. Hence, although prazosin is said to be primarily

an α_1-adrenergic antagonist, it is officially classified as an inverse agonist at the various α_1-receptor subtypes (IUPHAR, 2005), and further acts on the α_{2b}- and α_{2c}-adrenergic receptors (Bylund, 1988; Stone et al., 2007). Similarly, clonidine also acts as an agonist at α_{1d}-adrenergic receptors (IUPHAR, 2005). Timing of the drug administration after nerve injury is important as well, although it is difficult to compare across injuries which may have different mechanistic time-courses. As mentioned above, the influence of SNS may change over time, for example with sympathetic sprouting, or differential expression of various adrenergic subtypes in particular tissues, that develop at varying times after the different types of injuries. Nevertheless, a role for the α_2-receptors in mediating NE effects in peripheral tissues is likely important.

In relation to vasculature, it is well known that α_2-adrenergic receptors can have a vasoactive action on receptors at smooth muscle cells, as well as reducing NE release by acting at autoreceptors on SPGNs. As for results in studies of afferent nerve activity, contradictory results exist with some studies showing that α_2-receptor activation leads to vasoconstriction (Chotani et al., 2000), while others showing vasodilatation (Bockman et al., 1993). As discussed in chapter 1, while non-specific α_1/α_2-adrenergic receptor activation by NE is vasoconstrictive, vasoactive effects of individual α-adrenergic receptor subtypes can be strongly influenced by species, expression of subtypes in particular vascular beds, and the specificity of the drugs used to activate particular α-adrenergic subtypes. In the rat cremaster muscle preparation, it has been reported that activation of α_2-adrenergic receptors produces particularly significant vasoconstrictive effects in the more distal small pre-capillary arterioles than in the larger arterioles (Faber, 1988; Ohyanagi et al., 1991). This may be particularly relevant to I-R injury which has significant effects within the microvasculature.

Apart from direct action on SPGN or smooth muscle adrenergic receptors, it has also been proposed that α_2-receptor activation causes NO release within vascular endothelial cells. The vasodilatation induced by α_2-receptor activation has been shown to be blocked by NOS inhibitors in various tissues and species

(Bockman et al., 1996; Figueroa et al., 2001; Molin & Bendhack, 2005). Further, phentolamine, yohimbine, rauwolscine, and idazoxan vasodilatory actions have been shown to be reversed by NOS inhibitors in endothelial vasculature (Traish et al., 1998; Kim et al., 1999b). Interestingly, a recent study in human volunteers showed that application of low doses of topical clonidine leads to vasoconstriction, but higher doses induces pronounced vasodilatation that is blocked by NOS inhibitors (Hermann et al. 2005). However, while it is unclear how this translates to other species and to pathological conditions, a vasoactive role for peripheral α_2-adrenergic activation is implicated and likely mediates NE effects in a significant number of conditions. In CPIP rats, as discussed in chapter 4, we found that both an intradermal α_1-adrenergic receptor antagonist, an α_2-adrenergic antagonist, and a NO donor were able to inhibit NE-induced nociceptive behaviours. Close arterial injection of both adrenergic antagonists attenuated NE-induced reduction in blood flow, suggesting all these agents acted to inhibit NE vasoconstriction, which is likely due to both α_1- and α_2- adrenergic receptor activation on smooth muscle cells. This is consistent with old studies in rabbit and rat hind limb which show that both prazosin and yohimbine antagonize NE-induced vasoconstrictions (Madjar et al., 1980; Hamed et al., 1986). It may seem surprising that yohimbine reduced NE-induced pain and reductions in blood flow, but was unable to reduce allodynia in CPIP rats. However, peripheral exogenous administration of NE (used for NE-induced pain and vasoconstriction studies) may produce large effects on vascular smooth muscle α_2-adrenergic receptors, which would over-ride any influence of the NE at α_2-adrenergic autoreceptors on SPGNs. In this way, yohimbine's actions at smooth muscle cells would be more important than its action at SPGN autoreceptors. However, no exogenous NE was used in the allodynia trials, and therefore yohimbine's effects at SPGN autoreceptors would be more prominent in this condition.

5.4 NE-induced pain and vasoconstriction: indirect sensory-afferent coupling

The results of chapter 3 and 4 propose that changes in the vasculature can contribute to mechanical allodynia in CPIP rats. Consistent with the vascular abnormalities and vasospasms known to occur after I-R injury, and the success of

vasodilators in relieving CPIP allodynia, we examined whether intradermal NE administration in the ipsilateral hind paw induces enhanced pain in CPIP rats. Animals with mechanical allodynia (responders) showed greater nociceptive behaviours in response to low doses of intradermal NE in the ipsilateral paw as compared to animals without mechanical allodynia (non-responders), while shams did not show any nociceptive behaviours. This is consistent with several studies demonstrating NE-induced pain after intradermal injection in CRPS-I patients, particularly in those with SMP (Torebjörk et al., 1995; Ali et al., 2000), but no spontaneous pain in healthy controls. There was also associated NE vasoconstrictor hypersensitivity in the ipsilateral paw in CPIP rats with mechanical allodynia, but not in rats without mechanical allodynia, or in sham rats. These results are also similar to findings of vasoconstrictive hypersensitivity that have been reported in CRPS-I patients (Arnold et al., 1993; Teasell & Arnold, 2004). Administration of NE to induce vasoconstriction may also be said to mimic a vasospasm, which appears more severe or more likely to occur in CPIP rats with mechanical allodynia.

Electrophysiological demonstrations of abnormal NE-induced activation of primary afferent nerves is traditionally described as evidence of "direct coupling" between sympathetic efferent and primary afferent nerves. As discussed in chapter 1, "direct coupling" may be due to processes such as sympathetic sprouting and/or upregulation of adrenoceptors on primary afferent fibers, although no studies have been able to conclusively show that such coupling affects pain behaviour. *In vivo* nerve recordings have shown increased neuronal activity in response to NE after nerve injury as discussed in chapter 1. However, NE-induced activation of primary afferent nerves may also be affected by intervening factors, for example inflammatory mediators, vascular changes or other mechanisms of "indirect coupling" (Rubin et al., 1999; Jänig & Baron, 2003).

Old studies using electrophysiological recordings in neuromas and DRG of nerve transected animals had found that in addition to a significant proportion of nerves exhibiting hypersensitivity to NE, an even greater percentage responded

to hypoxia. These effects were absent in normal animals (Burchiel, 1984) Vasoconstrictor activity in cutaneous tissues and the hyperexcitability of C-fibers in response to hypoxia are also known to increase in cats with peripheral neuromas (Blumberg & Jänig, 1985; Häbler et al., 1987). It has also long been known that after brief tourniquet ischemia and reperfusion in humans, there is spontaneous firing of nociceptive fibers that correlate with paresthesia (Ochoa & Torebjörk, 1980). More recently, Häbler et al. (2000) found that DRG cells in SNL rats increased their firing rate not only when exposed to NE, but also when exposed to non-adrenergic vasoconstrictors, and further found that changes in neuronal firing correlated with DRG blood flow. These results suggest that ischemia or vasoconstriction can produce an indirect coupling of sympathetic and sensory neurons. In CPIP animals, vascular hypersensitivity to NE after I-R injury can be hypothesized to maintain vasospasms and chronic ischemia contributing to pain and exacerbation of ischemic pathology. In allodynic CPIP rats, it is possible to mimic NE-induced nociceptive behaviours with non-adrenergic vasoconstrictors, or with a NOS inhibitor, further suggesting a vasoactive pain mechanism for NE-induced pain in this animal model. Our finding that a NO donor vasodilator counteracts NE-induced nociceptive behaviours also supports this hypothesis. The idea that intervening vasoconstriction is involved in "coupling" may be particularly relevant to an animal model of CRPS-I, which is not expected to involve a major nerve injury. We demonstrated there is a correlation between NE-induced nociception and alterations in NE-induced vasoconstriction in CPIP rats providing evidence that indirect coupling mediated by the vasculature might underlie nociception in these animals.

The results of our studies suggest that adrenergic vasoconstrictor hypersensitivity may be a mechanism maintaining ischemic pain and CPIP mechanical allodynia. Chronic tissue ischemia and no-reflow after I-R injury (Laferrière et al., 2007) may prolong the action of either exogenously administered or endogenously released NE resulting in vasospasms and pain. Anti-sympathetic agents and vasodilators may also be effective anti-allodynic treatments by increasing the clearance of NE from the ischemic hind paw. I-R

injury can also directly affect NE action. Three hour ischemia and reperfusion of the canine femoral artery induces NE vasoconstrictive hypersensitivity starting at 1 hour post-reperfusion in isolated arteries. Binding studies suggest that it is due to an increased density of α-adrenoceptors on vasculature smooth muscle cells (Sapienza et al., 1996). Even one-hour of ischemia of rodent tail artery induces NE vasoconstrictive hypersensitivity starting at 1 hour post-reperfusion. While vascular adrenergic receptors were not observed to be upregulated in this study, there was increased coupling of these adrenoceptors to their G-proteins (Seasholtz et al., 2001). Changes in adrenergic receptors on primary afferent neurons or sympathetic efferent fiber sprouting after nerve injury are normally detected several days or weeks after initial injury, and do not always coincide with painful symptoms (Cho et al., 1997; Birder & Perl, 1999; Yen et al., 2006). Unlike rodent nerve injury models, which often take at least a few days to show prominent nociceptive behaviours, we showed in Chapter 2 that CPIP rats display nociceptive signs by 8 hours post-reperfusion. Subsequently, we have found that some CPIP rats exhibit allodynia as early as 2 hours post-reperfusion (unpublished results). This suggests that nociception may be more likely to correlate with NE vasoconstrictive hypersensitivity that can occur within hours of ischemia. Together with the finding that adrenergic vasoconstrictor responses and nociceptive sensitivity to NE is significantly greater in animals with mechanical allodynia ("responders"), than in those without allodynia ("non-responders) controls, the above findings provide further evidence that vasoconstrictor hypersensitivity may be a relevant pain mechanism after I-R injury in CPIP rats. Vascular hypersensitivity to NE has also been reported in CCI rats at three weeks post-injury (Kurvers et al., 1998).

5.5 The role of free radicals and nitric oxide in CPIP

Since oxidative stress is a prominent part of I-R injury, and initiates many of the pathological consequences, we hypothesized that decreasing free radicals will promote antinociception in CPIP rats. Single-dose trials with an antioxidant NAC (500 mg/kg i.p.) and a free-radical scavenger TEMPOL (250 mg/kg i.p.) showed particular effectiveness in reducing mechanical allodynia. The doses used

did not induce rotorod abnormalities, and we have subsequently seen dose-dependent effects with lower doses of both these agents (unpublished observations). Naik et al. (2006a) showed that 30-300 mg/kg doses of NAC reduced nociception in CCI rats, with the higher doses also reducing mechanical hyperalgesia, cold allodynia, and heat hyperalgesia. TEMPOL at doses of 100 mg/kg also reduces heat hyperalgesia in CCI rats (Tal, 1996). At this dose range, these agents also produce anti-nociceptive effects in the formalin test (Hacimuftuoglu et al., 2006), reduce heat hyperalgesia in carageenan inflammation (Khattab, 2006), and decrease secondary mechanical hyperalgesia induced by capsaicin (Lee et al., 2007). Hence, decreasing free radicals with these drugs is antinociceptive in various animal models, although we hypothesize that this may be particularly relevant after I-R injury.

The antioxidant and free radical scavenger we used are known to have other mechanisms of action which influence nociception depending on the animal model. For example, although NAC is primarily a glutathione precursor, it is also known to have anti-inflammatory effects (Geudens et al., 2008) and improves blood flow via effects on NO and α-adrenergic receptors (Girouard et al., 2003). Similar effects have been noted with TEMPOL: anti-inflammatory (Thiemermans, 2003), vasodilatory effects induced by promoting NO release (Zöllner et al., 1997), and anti-sympathetic effects (Xu et al., 2001). The broad action of these free radical inhibitors makes it difficult to ascertain one mechanism of action responsible for their anti-allodynia effects in CPIP rats. It is probable that these additional effects may be secondary to reducing free radicals. Furthermore, these additional actions may be particularly useful for CPIP rats; reducing inflammation and improving tissue perfusion can alleviate the deleterious effects of I-R injury and thereby reduce CPIP mechanical allodynia.

In chapter 3, we theorized that anti-allodynic effects of anti-sympathetic agents in CPIP rats were related to their vasodilatory effects. In support of this, we found that a NO donor, SIN-1, also reduced mechanical allodynia in CPIP rats. In chapter 4, another NO donor SNP was co-administered with NE intradermally and was shown to attenuate NE-induced nociceptive behaviours.

Therefore, it appears that either systemically or locally administered NO donors are antinociceptive in CPIP rats. In the literature, however, the role of NO in other pain models has been inconsistent, with evidence for both pro-nociceptive and anti-nociceptive effects.

In chapter 3, the anti-allodynia effect of i.p. SIN-1 was tested at 20 minutes post-drug administration. Central pro-nociceptive mechanisms of NO donors which show a delayed onset, as discussed in chapter 1, are not likely to be involved here. Peripheral mechanisms such as vasodilatation occur immediately with entrance of NO into the circulation and should quickly affect ischemic tissue. In CPIP, NO donors are hypothesized to function as anti-nociceptive agents due to their prominent vasodilatory properties. This mechanism is likely to be particularly important after I-R injury in order to alleviate vasospasm, no-reflow, and potentially vascular hypersensitivity to vasoconstrictors such as NE as found in chapter 4. However, increased NO is also known to be deleterious in I-R injury due to its ability to combine with superoxide free radical and production of toxic products such as peroxynitrite (Pacher et al., 2007). Several studies have shown that reducing NO with non-specific NOS inhibitors such as L-NAME can improve muscle contractility after skeletal muscle and hind limb ischemia of 30 minutes to 3 hours in rats and mice (Knight et al., 1997; Ikebe et al., 2002; Zhang et al., 1997). Improved muscle viability and contractility is also reported with inducible nitric oxide synthase (iNOS) inhibitors such as 1400W and early treatment with eNOS inhibitor such as L-NIO in 2-3 hour skeletal muscle ischemia (Phan et al., 1994; Patel et al., 2004). Interestingly, the combination of the iNOS inhibitor 1400W together with the NO donor SNAC has been found to be more effective than either drug alone in improving muscle viability at 3 hours, 24 hours, and 7 days after a 3-hour rat hind limb skeletal muscle ischemia (Barker et al., 2005). Studies in hind limb I-R injury models in rabbits and rats have directly measured NO levels, and shown that while there is an initial NO increase during ischemia, it then consistently decreases below baseline and even further with reperfusion (Brovkovych et al., 1999; Hällstrom et al., 2002; Kubant et al., 2006). The decreased NO has been shown to correlate with increased superoxide

and peroxynitrite levels, as well as vasoconstriction/vasospasm and interstitial oedema (Huk et al., 1997; Kubant et al., 2006; Stoffels et al., 2007). The reperfusion response observed in CPIP is consistent with the I-R injury literature with the conversion of NO to free radicals during the hyperaemic period which also involves inflammatory processes, hence the appearance of the "hot" hind paw in CPIP. With the hyperaemia subsiding, the subsequent reduction of NO leads to prolonged vasoconstriction and vasospasm. In CPIP rats, hyperaemia has subsided at 4 hrs post-reperfusion and the paw is clearly ischemic by 2 days (Laferrière et al., 2007).

As discussed above and in chapter 1, pathological consequences of hind limb I-R injury can be prevented early by NOS inhibitors which will inhibit formation nitrogen free radicals, or later by NO donors that will inhibit vasospasms. Consistent with this, in our studies, NO exogenously administered at 2 days post-reperfusion is more likely acting by restoring the decreased NO that has been converted to free radicals, and reversing vasospasms to improve blood flow. It can attenuate mechanical allodynia when administered systemically, but also reverse NE-induced nociceptive behaviours when administered directly into the hind paw. Hence, it can be hypothesized that the vasoactive role of NO donors is particularly important in attenuating allodynia associated with I-R injury. On the other hand, at this time point, administration of eNOS inhibitors is more likely to further inhibit endothelial-derived NO and primarily promote vasoconstriction (Rees et al., 1990), rather than acting to reduce conversion of NO to free radicals. eNOS is generally thought to be primarily upregulated only during ischemia and early after reperfusion (Messina et al., 2000; Khanna et al., 2005). As reported in chapter 4, we found that intradermal administration of L-NIO at 2 days post-reperfusion induced significant nociceptive behaviours in CPIP rats, similar to the effects of intradermal NE. Our studies are the first reports of using NO donors as anti-nociceptive agents, and NOS inhibitors to induce nociceptive behaviours, after experimental I-R injury. Based on our studies in CPIP rats that showed successful anti-allodynia with anti-sympathetic treatments and NO donors, it

would be tempting to speculate that vasodilators may be most effective in CRPS patients that also respond to anti-sympathetic treatments.

5.6 Nerve injury and CRPS-I

The differentiation between type I and type II CRPS is defined by the presence of nerve injury as indicated by the majority of researchers and clinicians, including in many of the papers discussed in this thesis. However, CRPS-I is considered one of the least understood and most controversial pain syndromes, and there are historical (Schott, 2007), as well as recent ongoing, debates in the literature whether CRPS-I is better classified as a neuropathic, CNS, autonomic or psychiatric disorder (Oaklander et al., 2006; Jänig & Baron, 2006; Ochoa, 2006; Rocco & Raymond, 2006). As discussed in chapter 1, new studies report distal nerve injury and altered cutaneous innervations in CRPS-I patients (Oaklander et al., 2006; Albrecht et al., 2006). The proposition that CPIP is an animal model of CRPS-I, and not of CRPS-II, has been based on observations that the I-R injury used does not cause a major nerve injury. This is consistent with clinical identification of CRPS-I (and not of type II), which is diagnosed by excluding the presence of a major nerve injury using nerve conduction velocity (Manning, 2000). Light microscopy studies did not reveal nerve degeneration under the tourniquet and sural nerve conduction velocity is normal in CPIP rats.

A couple of studies have directly targeted nerves in attempts to create animal models of CRPS-I. Electrical sciatic nerve stimulation was first said to induce a syndrome similar to CRPS-I (Vatine et al., 1998) with heat hyperalgesia and cold allodynia persisting for 3 weeks, and mild mechanical allodynia for 1 week post-stimulation. Other abnormalities typical of CRPS-I, however, such as inflammation or ischemia were not noted. It is difficult to speculate on the clinical correlate of electrical stimulation and further studies have not been reported using this method. Nevertheless, this study suggests that excessive nerve stimulation can induce persistent nociception. A more recent study induced a minor distal nerve injury in the tibial nerve by needle stick (Siegel et al., 2007). This resulted in significant pain behaviours for at least 7 days which subsided by 14 days, as well as prominent oedema. There was no reduction in nerve conduction velocity,

similar to that observed in patients with CRPS-I. This study used different size needles to evoke injury, and found no correlation between painful symptoms and needle size. Like Vatine et al. (1998), it is important in promoting the idea that CRPS-I can involve, excessive nerve activity or minor nerve injury, which may be undetectable in the clinic, or not recognized to occur at the time of initial injury. However, ischemic changes were not found in this model, as in CPIP and other animal models of CRPS-I discussed above. Further characterization would be useful to differentiate this model from other nerve injury models and animal models of CRPS-I.

Recently, it was found that there is a decrease in small fiber innervations in the skin of CPIP rats (Laferrière et al., 2007) and these findings are paralleled by clinical investigations showing reduced small fiber density in CRPS-I patients (Oaklander et al., 2006). It has been shown that three to four hours of complete nerve ischemia can result in endoneurial oedema and significant nerve fiber degeneration (Nagamatsu et al., 1996), peaking several days post-reperfusion (Saray et al., 1999; Iida et al., 2003), while 5 hours of ischemia result in severe demyelination (Nukada et al., 1997). It is likely that three hours of hind paw ischemia may produce a certain degree of small or reversible nerve injury. Although Schoen et al. (2007) reported that 3 hours of hind limb ischemia produced signs of nerve injury, the mechanical allodynia in these rats could be reduced by ischemic preconditioning, a process that ameliorated ischemic pathology, such as reduced capillary density, but not the nerve injury. Hence, the appearance of nerve injury after tourniquet I-R injury may not necessarily be related to pain. This is consistent with the potential for nerve injuries to also produce painless neuropathies (Wall, 1991). More research is needed with CPIP rats to determine the degree of nerve injury using electron microscopy not only under the tourniquet, but also in the ischemia limb, and at various time points post-injury to determine the possibility and the significance of a secondary nerve injury. It can further be speculated that ischemic and neuropathic components can co-exist, and the difference in effectiveness of anti-sympathetic agents between 2 and 7 days post-reperfusion may be due to changing mechanisms that maintain

mechanical allodynia. In CPIP, it is likely that three hours of tourniquet ischemia induces CPIP pain by a combination of mechanisms involving chronic tissue ischemia (due to vasospasms and no-reflow), and potentially secondary nerve injury that evolves over time. Future studies will help define the spectrum of pain mechanisms that may be present in CPIP rats. CPIP remains the first systematic study measuring persistent nociception in an animal model after hindpaw tourniquet I-R injury, and one of the few chronic pain animal models developed that is being increasingly characterized specifically to better understand CRPS-I.

5.7 Limitations and future directions

This thesis presents a novel animal model of chronic pain and ideas for pain mechanism that may be translatable to the human condition of CRPS-I. It is important to highlight some limitations in the results and interpretations of results presented in this thesis. Some of these can also suggest potential future directions for follow-up.

As discussed throughout the thesis, the route of administration used in our studies influences the mechanism of action of drugs used in our studies. The i.p. route used for some of the drugs suggests bloodstream distribution and central effects if the drug crosses the blood-brain barrier. While guanethidine is known not to cross blood-brain barrier, prazosin, yohimbine, SIN-1, and clonidine do. As discussed, central effects on the α_2-adrenergic receptor are likely, while this is hypothesized not to be the case for NO donors. The intradermal and intra-arterial routes used in our studies are thought to target primarily peripheral mechanisms. The close-arterial injections of NE and adrenergic antagonists will primarily affect the peripheral paw tissues, although the drugs will continue to the veins and be diluted and eventually cleared in the systemic circulation. To control for systemic effects, in laser Doppler studies we used an i.v. NE injection protocol to allow simultaneous measurement in both hind paws, and found greater NE-induced vasoconstriction in the ipsilateral versus contralateral hind paw, suggesting there were specific alterations in the ipsilateral hind paw. For intra-dermal injections, we used low doses and low volumes and a small needle size to optimize delivery. However, it may have been useful to inject the same

intradermal doses to the contralateral hind paw to examine potential central effects. It should be noted that some studies have found different pro-nociceptive and anti-nociceptive effects of NO donors depending on whether intradermal or s.c. injection is used (Vivancos et al., 2003). In a preliminary study, we found a partial reversal of mechanical allodynia after intradermal injection of SNP (Xanthos & Coderre, 2005) which is consistent with the vasoactive role of NO donors hypothesized.

One unavoidable limitation in our studies is the use of anaesthetics in the laser Doppler studies, which can limit conclusions on parallels between behaviour and blood flow measures. We chose urethane for anesthesia in our laser Doppler studies to reduce confounds with vasoconstrictor responses in the rat (Le Noble et al., 1987; Kwolek et al., 2005). Urethane is a widely used anaesthetic agent that allows prolonged and consistent anesthesia. It would be interesting for future studies to include other techniques that would be feasible in awake animals, or in isolated tissues where anaesthetics are not needed. For example, thermography and laser Doppler imaging may be performed in awake restrained CPIP rats after intradermal injections of NE in the paw, and compared to shams and non-responders. Further, it may be interesting to assess whether CPIP animals show enhanced nociceptive behaviours after manipulations that activate the SNS centrally (to model human studies reviewed in chapter 1). In addition, animals with chronic SNS abnormalities, for example spontaneously hypertensive rats, may be tested for altered nociceptive behaviours in CPIP.

During pilot studies, a 3-hour time-course of ischemia for CPIP was determined to be the optimal duration that induced mechanical allodynia in a significant number of animals. In order to avoid long-term complications, clinical recommendations for a safe duration of ischemia during surgery is two hours. Data from experimental I-R injury literature discussed in chapter 1 suggests that ischemic durations of greater than 3 hours will induce irreversible muscle and nerve damage. Somogyi & Selye (1969) had used a three-hour tourniquet in rats and found that it produced an exaggerated hyperaemic response that appeared to resemble the “hot” phase of CRPS-I patients. It would be interesting in future

studies to systematically measure different durations, as well as different degrees of ischemia, in order to gain an exact understanding of parameters that will induce optimal conditions for the CPIP model and to mimick CRPS-I without causing a major nerve injury.

Since not all rats developed allodynia in response to the I-R injury, we decided to test only rats with von Frey scores below 6 grams (approximately 70% of rats). This technique was used so that we would avoid testing the anti-allodynic effects of test agents in rats that were not allodynic. While this selection method may have been non-standard in comparison with other animal models of chronic pain, it is similar to clinical studies in which patients with injuries that do not have pain are routinely not used in analgesic trials. Furthermore, in chapter 4, we exploited the "responder" and "non-responder" classifications and actually found differences in their response to NE, although we excluded rats with thresholds between 6 and 10 (only about 10% of rats). We use von Frey thresholds to make this classification between "responders" and "non-responders", since it was the most robust nociceptive measure in these rats. In chapter 3 and 4, we did not generalize our conclusions beyond mechanical allodynia. However, CRPS-I and neuropathic pain patients show a variety of different sensory abnormalities and spontaneous pain. Hence, defining pain or nociception as related to SMP and CRPS based on only one sensory abnormality may be limit the ability to translate our findings to the clinical condition. Testing multiple modalities of CPIP pain (cold allodynia, mechanical hyperalgesia, etc) simultaneously should be attempted in future pharmacological studies. Since we detected prominent tactile allodynia and cold allodynia, but not heat hyperalgesia, it would be interesting to correlate this by studying tactile-responsive Aβ-fibers (Woolf & Doubell, 1994) and heat responsive C-fibers (Perl, 1996) in CPIP animals. Futher, studies examining heat-sensing TRP channels of the vanilloid family (Nakagawa & Hiura, 2006) may be expected to show little abnormal activity in the CPIP model, while cold-sensing channels such TRPM8 (Fleetwood-Walker et al., 2007) may be particularly important and may be targeted by novel pharmacological agents.

Although our studies in chapter 4 indicated the CPIP responders displayed both NE-induced pain and adrenergic vasoconstrictor hypersensitivity, we did not determine whether vascular hypersensitivity could be extended to other vasoactive agents. It would be interesting to clarify whether this vasoconstrictor hypersensitivity is indeed specific to adrenergic mechanisms. Based on the ability of vasopressin and L-NIO to mimic the behavioural nociceptive effects of NE, we can also expect parallel vascular hypersensitivity to other vasoconstrictors, for example vasopressin, angiotensin II, or endothelin. Further, as discussed above, vascular abnormalities such as no-reflow may also be responsible for a prolonged action of NE in peripheral tissues. This may then not be related to adrenergic vascular hypersensitivity as hypothesized, but rather other factors such as reduced NO-dependent vasodilatation. As discussed in chapter 1, various types and degrees of I-R injuries can lead to endothelial dysfunction which can show altered vasoconstriction and vasodilatation to a variety of agents. On the other hand, adrenergic vasoconstrictive hypersensitivity observed may indeed be due to various mechanisms such as an upregulation of specific subtypes of adrenergic receptors or enhanced G-protein coupling at particular adrenoceptors. Future studies may clarify these phenomena by examining changes in the expression of α_1- and α_2-receptor subtypes in vasculature and other peripheral tissues, alterations in forskolin-stimulated cAMP in hind paw tissues, as well as the effect of other vasoactive agents on blood flow in CPIP animals.

Another limitation in our interpretations from chapter 3 is that CPIP animals show evidence of SMP, although guanethidine treatment and phentolamine did not result in full reversal of mechanical allodynia. Therefore, it would be important for future studies to assess the anti-nociceptive effects of other sympathectomy methods such as surgical sympathectomy or sympathetic ganglion blocks in CPIP rats (Park et al., 2000). Other drugs more selective for α_1- and α_2-adrenergic receptors, such as terazosin or atipemazole (which have been demonstrated to modulate nociception), could also be used to further confirm the actions of prazosin and yohimbine at those receptors and confirm the mechanisms suggested for anti-sympathetic effects. A preliminary study in our

lab that examined more specific α_1-subtype (a, b, d) and α_2-subtype (a, b, c) adrenergic antagonists administered systemically on mechanical allodynia in CPIP rats, but did not find any significant anti-allodynic effects (unpublished observations), although more studies are needed to determine appropriate dosages, selectivity of agents, and effects of these agents on various pain modalities. Also, it is possible that a less selective drug is more efficacious, and studies should examine combinations of these selective agents. In addition, the SNS is known to release other chemical mediators that are important in pain such as neuropeptide Y, adenosine triphosphate, prostaglandins and adenosine (Rudehill et al., 1992; Burnstock, 1995; Janig et al., 1996; Liu et al., 2004). Hence, anti-sympathetic and anti-adrenergic treatments used in our studies may not be able to fully inhibit SNS activity, as would a surgical sympathectomy. However, a partial anti-nociceptive effect of anti-sympathetic agents may be fully consistent with CRPS-I, in that some patients show pain relief with anti-sympathetic agents (SMP), while others do not (SIP), and probably relevant to the likelihood that multiple pain mechanisms are involved in maintaining CPIP pain.

In order to further understand the differences between CRPS-I and CRPS-II, it is important that future studies compare the findings of CPIP rats to other commonly used animal pain models. Studies done in parallel with the well characterized nerve injury models such as CCI, SNL, or SNI, and with the other animal models of CRPS-I, will elucidate the similarities and differences in their underlying mechanisms. Future studies should also put this animal model into a broader context by considering in what ways the pain mechanisms in this animal model can be relevant to pain mechanisms of other disease states that display vascular pain and ischemia, such as Raynaud's syndrome, intermittent claudication, peripheral vascular disease, critical limb ischemia, diabetic neuropathy and angina. Most importantly, studies with CPIP in parallel with clinical research on CRPS-I will further help understand both conditions and allow for the translation of animal research to the understanding of the clinical condition. Multi-disciplinary perspectives both at the basic level and clinical level will help in better understanding both basic mechanisms and clinical treatments.

5.8 General conclusions

In conclusion, this thesis describes the first systematic research measuring nociception, and elucidating relevant pain mechanisms, after a rodent hind paw I-R injury. This animal model is hypothesized to be particularly relevant to the clinical condition of CRPS-I, and results in this thesis are consistent with the hypothesis. Contributions of the SNS and adrenergic vasoconstrictor hypersensitivity to pain mechanisms have been examined in this model and related to the clinical condition. Anti-sympathetic and vasodilatory drugs show effectiveness in reducing mechanical allodynia in this animal model, and animals with mechanical allodynia show adrenergic nociceptive and vasoconstrictor hypersensitivity, which is thought to relate to SMP in CRPS patients. It is hoped that this animal model will help improve understanding of the pain mechanisms following I-R injury and in CRPS-I patients. With further characterization, this animal model should become a useful and powerful tool which should help bridge the divide between basic research and chronic pain in humans. Increased understanding coupled with awareness of ischemic processes and vascular abnormalities contributing to pain are hoped to help improve and generate more effective therapies for CRPS.

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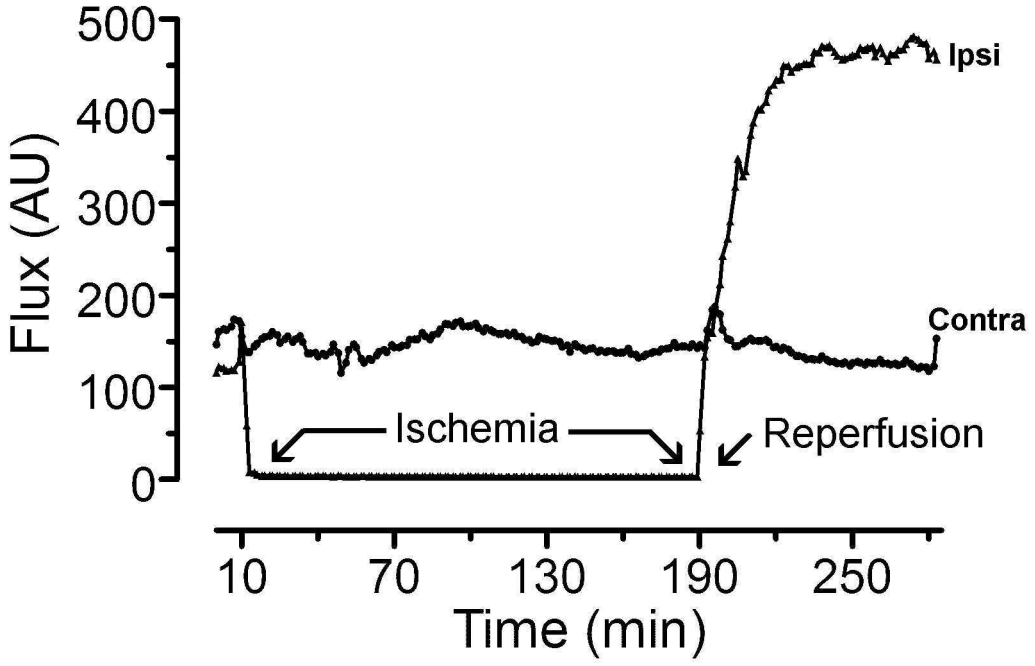
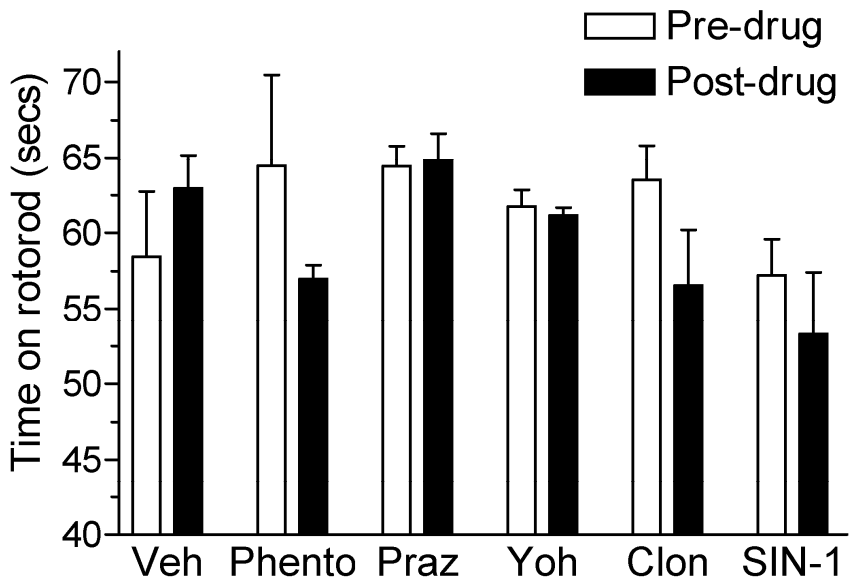
Appendix A



Blood flow measures (arbitrary flux units) with simultaneous measures of the ipsilateral and contralateral hindpaw in a rat undergoing CPIP I-R injury. The O-ring was placed at the 10 minute timepoint and resulted in ~100% decrease blood flow in the ipsilateral hindpaw, while no change in the contralateral hindpaw. The O-ring was removed at the 190 minute timepoint resulting in a significant and persisting hyperemia approximately three times the baseline in the ipsilateral hindpaw, with only a minor transient change in blood flow on the contralateral hindpaw.

Appendix B



Time spent on an accelerating rotorod before (Pre-drug) and after (Post-drug) systemic treatment with VEH (n = 11), phentolamine (10 mg/kg) (n = 6), prazosin (Praz, 5 mg/kg) (n = 6), yohimbine (Yoh, 5 mg/kg) (n = 6), clonidine (Clon, 0.1 mg/kg) (n = 6) and SIN-1 (10 mg/kg) (n = 6). Two-way ANOVA revealed non-significant main effects of treatment ($F_{5, 81} = 1.18$; $P > 0.05$) and time ($F_{1, 81} = 2.32$; $P > 0.05$ (pre/post), and a non-significant interaction ($F_{5, 81} = 1.54$; $P > 0.05$).

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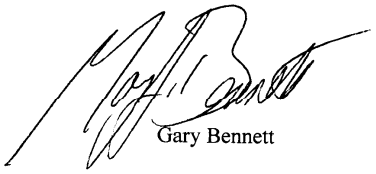
April 18, 2008

I, Gary Bennett, as co-author of the two articles "Norepinephrine-induced nociception and vasoconstrictor hypersensitivity in rats with chronic post-ischemia pain" and "Chronic post-ischemia pain (CPIP): a novel animal model of complex regional pain syndrome-type I (CRPS-I; reflex sympathetic dystrophy) produced by prolonged hindpaw ischemia and reperfusion in the rat", both published in the journal "Pain" in 2008 and 2004, respectively, give my permission to Dimitris Xanthos to include the manuscripts as part of his doctoral dissertation.



Gary Bennett

Appendix D

CEN

Guidelines for completing the form are available at www.mcgill.ca/researchoffice/compliance/animal/forms

McGill University
Animal Use Protocol – Research

For Office Use Only:
Protocol #: 4787
Approval End Date: Sept 30, 2008
Facility Committee: DCW

Title: Pain mechanisms in an animal model of CRPS-I; AND Structural and functional brain imaging in animal models of persistent pain
(must match the title of the funding source application)

☐ **New Application** ☒ **Renewal of Protocol #** 4787 ☐ **Pilot** **Category** *(see section 11)*: D

1. Investigator Data:

Principal Investigator: Terence Coderre **Phone #:** 5773
Unit/Department: Anesthesia Research Unit **Fax#:** 8241
Address: Room 1203 McIntyre Bldg. **Email:** terence.coderre@mcgill.ca

2. Emergency Contacts: Two people must be designated to handle emergencies.

Name: Terence Coderre **Work #:** 5773 **Emergency #:** (514) 928-5773
Name: Andre Laferriere **Work #:** 2903 **Emergency #:** (514) 384-2436

FC ove

3. Funding Source: 17818

External ☒ 37004
Source (s): CIHR 36573
Peer Reviewed for the project proposed in this Animal Use Protocol: ☒YES ☐ NO**
Status : ☐ Awarded ☒ Pending
Funding period: 04/2008-03/2011

Internal ☒
Source (s): Edwards & Stairs Foundations
Peer Reviewed: ☒ YES ☐ NO**
Status: ☒ Awarded ☒ Pending
Funding period: 07/2007-08/2008

For Office Use Only:

ACTION	✓	DATE
CCs		
DB	✓	NOV 1807
APPROVED		

** All projects that have not been peer reviewed for scientific merit by the funding source require 2 Peer Review Forms to be completed e.g. Projects funded from industrial sources. Peer Review Form at www.mcgill.ca/researchoffice/compliance/animal/forms

Proposed Start Date of Animal Use (d/m/y): ____ **or ongoing** ☒

Expected Date of Completion of Animal Use (d/m/y): ____ **or ongoing** ☒

Investigator's Statement: The information in this application is exact and complete. I assure that all care and use of animals in this proposal will be in accordance with the guidelines and policies of the Canadian Council on Animal Care and those of McGill University. I shall request the Animal Care Committee's approval prior to any deviations from this protocol as approved. I understand that this approval is valid for one year and must be approved on an annual basis.

Principal Investigator's signature: [signature] **Date:** 31/08/07.

Approved by:

Chair, Facility Animal Care Committee:	[signature]	**Date:** 8 Nov 2007
RESEARCH ETHICS OFF.	[signature]	**Date:** NOV-17'07
Chair, Ethics Subcommittee (as per UACC policy): Not Needed	—	**Date:** —
Approved Animal Use	**Beginning:** Oct-1, 2007	**Ending:** Sept 30, 2008

☐ This protocol has been approved with the modifications noted in Section 13.

July 2007

Received Sept 10/07

12 NOV. 2007