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Experimental studies on the mechanisms and treatments of chronic pain

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Karolinska Institutet

Stockholm 2014

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ISBN 978-91-7549-722-8

Printed by [Perssons Offsettryckeri AB]
Gårdsvägen 4
169 70 Solna

Experimental studies on the mechanisms and treatments of chronic pain

THESIS FOR DOCTORAL DEGREE (Ph.D.)

AKADEMISK AVHANDLING
som för avläggande av medicine doktorsexamen vid Karolinska Institutet offentligen försvaras på engelska i Hörsal, Nanna Svartz väg 2

Fredagen, den 5 December, 2014, kl 10:00

av

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Human beings are members of a whole,
In creation of one essence and a soul,
If one member is afflicted with pain,
Other members uneasy will remain,
If you've no sympathy for human pain,
The name of human you cannot retain.
(Saadi 1184-1283)

To my parents and Yang

ABSTRACT

Chronic pain is a major concern for physical and mental health of a large patient population today while casting a significant economical burden on society. Work presented in this thesis deal with aspects of mechanisms and treatments of chronic pain using experimental models.

A common characteristic for many chronic pain conditions, particularly those after nerve injury, is hypersensitivity to cold stimulation. In the first part of the thesis, I presented a new method using a Peltier thermode to examine the responses of rats to quantitative thermal stimulation (heating and cooling). Using this method with temperature as end points, I showed that we can reliably detect cold hypersensitivity in spinally injured rats as well as study quantitatively the effects of analgesics again cold pain.

Sinomenine is a morphinan derivative alkaloid originally isolated from the root of the climbing plant *Sinomenium Acutum* that is native to Japan and China. The root of *Sinomenium Acutum* has long been used in East Asia as a remedy for disease conditions similar to rheumatism and sinomenine is currently used in China as an anti-rheumatic agent. In the second part of the thesis, we characterized the analgesic effect of sinomenine in a variety of experimental pain models. We showed that while sinomenine has modest effects on acute pain in normal rats, it produces marked analgesic effects in a wide-spectrum of models, including neuropathic pain in rats and mice after injury to the peripheral and central nervous system, acute inflammatory pain by carrageenan in mice as well as arthritic pain in mice using the collagen antibody-induced arthritis model (CAIA). We further showed that under chronic administration, sinomenine maintained its analgesic effect in neuropathic and arthritic pain models without producing tolerance or dependence. Our results thus suggested that sinomenine may be considered as a novel analgesic in treating neuropathic and arthritic pain.

One of the main clinical features of rheumatoid arthritis (RA) is sex difference in its prevalence and symptoms, including pain. The underlying mechanisms of sex differences in RA are still largely unknown. In the last part of the thesis, we studied sex differences in the development arthritis and pain-like behaviors in mice using the CAIA model. We observed a significant sex difference (females > males) in the development of joint inflammation and localized mechanical allodynia in the paws after CAIA in CBA strain of mice. Similarly, female CAIA mice also developed more persistent spread mechanical allodynia in their neck and flank areas. Following CAIA, the greater mechanical hypersensitivity in females was correlated to a higher expression of ionized calcium-binding adapter molecule 1, but lower expressions of activating transcription factor 3 and galanin, in dorsal root ganglion (DRG) compared with males. We conclude that sex differences in the CAIA model in CBA mice are similar to the clinical condition and sex dependent phenotypic changes in the DRG may be keys for the sex differences in RA and pain.

Key words: Sinomenine, Neuropathic Pain, Sex Difference, Arthritic Pain, Cold Pain, DRG, Spinal Cord.

LIST OF PUBLICATIONS

I. **Gao T**, Hao JX, Wiesenfeld-Hallin Z, Xu XJ. Quantitative test of responses to thermal stimulation in spinally injured rats using a Peltier thermode: a new approach to study cold allodynia. *J Neurosci Methods* 2013; 212:317– 321.

II. **Gao T,** Hao JX, Wiesenfeld-Hallin Z, Wang DQ, Xu XJ. Analgesic effect of sinomenine in rodents after inflammation and nerve injury. *Eur J Pharmacol* 2013; 721:5-11.

III. **Gao T,** Shi T, Wang DQ, Wiesenfeld-Hallin Z, Xu XJ. Repeated sinomenine administration alleviates chronic neuropathic pain-like behaviors in rodents without producing tolerance. *Scand J Pain* 2014; 5:249–55.

IV. **Gao T**, Shi T, Wiesenfeld-Hallin Z, Svensson CI, Xu XJ. The analgesic effect of sinomenine on experimental rheumatoid arthritis. Manuscipt submitted.

V. **Gao T**, Su J, Shi TJ, Hao JX, Hökfelt T, Wiesenfeld-Hallin Z, Svensson CI, Xu XJ. Sex differences in the development of arthritis and hypersensitivity in mice with collagen antibody-induced arthritis: correlation with phenotypic changes in dorsal root ganglia. Manuscript submtted.

Contents

LIST OF ABBREVIATIONS

AA	Adjuvant arthritis
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
ATF-3	Activating transcription factor 3
CAIA	Collagen antibody induced arthritis
cAMP	Cyclic adenosine monophosphate
GAP-43	Growth Associated Protein 43
CD	Cluster of differentiation
CGRP	Calcitonin gene related peptide
CIA	Collagen induced arthritis
CII	Type II collagen
CNS	Central nervous system
COX	Cyclooxygenase
DMARDs	Disease-modifying antirheumatic drugs
DMSO	Dimethyl sulfoxide
DRG	Dorsal root ganglion
ERs	Estrogen receptors
GABA	Gamma-aminobutyric acid
GFAP	Glial fibrillary acidic protein
HPRT	Hypoxanthine phosphoribosyltransferase
IASP	International association for the study of pain
Iba-1	Ionized calcium-binding adapter molecule 1
IBS	Irritable bowel syndrome
IL	Interleukin
INF-γ	Interferon gamma
i.p.	Intraperitoneally
i.t.	Intrathecally
i.v.	Intravenously
LPS	Lipopolysaccharide
MAD	Median absolute deviation
MMPs	Metalloproteinases
MSA	Modular Sensory Analyzer
NASIDs	Non-steroid anti-inflammatory drugs
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NMDA	N-Methyl-D-aspartate
NO	Nitric oxide
NOS	Nitric oxide synthase
NPs	Neuron profiles
OVX	Ovariectomy
PAG	Periaqueductal gray
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PGE2	Prostaglandin E2
PI	Propidium Iodide

PLSD	Protected Least Significant Difference
p.o.	Per os
p38MAPK	p38 mitogen-activated protein kinases
RA	Rheumatoid Arthritis
RTX	Resiniferatoxin
ROS	Reactive oxygen species
RVM	Rostral ventromedial medulla
s.c.	Subcutaneously
SCI	Spinal cord injury
SD	Sprague-Dawley
SEM	Standard error of the mean
SP	Substance P
SNI	Sciatic nerve injury
TMD	Temporomandibular disorder
TNF	Tumor necrosis factor
TRP	Transient receptor potential
WDR	Wide dynamic range
5-HT	5-hydroxytryptamine

1 INTRODUCTION

1.1 PAIN

1.1.1 Definition and classification

Pain is processed by a multilayered system consisting of sensory, cognitive and affective components to exert its alarm function (Woolf, 2004). As defined by the International Association for the Study of Pain (IASP), pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage (Merskey and Bogduk, 1994). Based on the causes, pain can be further classified into three major categories, which are nociceptive pain, inflammatory pain and neuropathic pain.

Nociceptive pain is the common discomfort we experienced from noxious stimuli that are, or will potentially be, tissue damaging. The nociceptive pain is essential for the survival of animals since it serves as a warning signal, which elicits a protective or defensive response (Scholz and Woolf, 2002). In most cases nociceptive pain is acute as it stops when removing the stimulus. Inflammatory pain is produced by events that associated with, for example, tissue injury, infections, burns and autoimmune diseases. Activation of localized and recruited inflammatory cells during inflammation results in the release of various inflammatory mediators including cytokines, growth factors, neuropeptides, kinins, purines, amines, prostanoids and protons (Boddeke, 2001; Manthy et al, 2002) that sensitize nociceptors (peripheral receptors respond to noxious stimuli), and increase the sensitivity of sensory neurons to noxious stimuli (Scholz and Woolf, 2002). Inflammatory pain can be both acute and chronic. Neuropathic pain is pain following a primary lesion or dysfunction in the peripheral or central nervous system (Merskey and Bogduk, 1994). In the majority of the cases, neuropathic pain is chronic, difficult to manage and associated with plastic changes in the nervous system (Hökfelt et al., 1994).

1.1.2 Noxious stimuli and pain transmission

The sensation of pain starts from the detection of noxious (mechanical, thermal or chemical) stimulus by peripheral nociceptors in the skin (Basbaum and Jessell, 2000). Nociceptors are sensitive nerve terminals, consisting of two main categories which are Aδ mechanical and C-polymodal (Meyer et al, 2008). Aδ fibers are thinly myelinated afferents that giving rise to sharp, pricking pain to mechanical or thermal stimulation at high intensity. C fibers are unmyelinated afferents with small diameters and lower conduction velocity, that responding to both mechanical and thermal stimuli at various intensities (D'Mello and Dickenson, 2008). Noxious stimuli are converted into electrical activities by nociceptors (Scholz and Woolf, 2002), and then transmitted into spinal cord via dorsal root ganglion (DRG) neurons, which synapse onto the dorsal horn neurons (Besson and Chaouch, 1987; Basbaum et al, 2009).

The primary afferents terminate in the dorsal part of the spinal cord following a highly organized style. Aδ fibers and C fibers predominately terminate in the superficial laminae I and II (Light and Perl, 1979), while large myelinated fibers such as Aα and Aβ fibers usually terminate more ventrally in laminae III and IV (Brown, 1981; Besson and Chaouch, 1987). Dorsal horn neurons responding to peripheral stimulation are generally classified into three types: low threshold neurons preferably activated by innocuous

stimuli, wide dynamic range (WDR) neurons, responding to a range of stimulation coming from thermal, chemical and mechanical modalities in a graded pattern with respect to the intensity of stimulation, and high threshold neurons that respond exclusively to noxious stimuli (Cervero et al, 1976; Besson and Chaouch, 1987).

When nociceptive inputs have been transmitted to the dorsal horn, signals are integrated at the spinal level and generated, often through specific interneuron mediated networks, local somatic or sympathetic reflexes. The nociceptive signal will also be further relayed to the medulla, brainstem and thalamus via ascending tracts. The thalamus is the region where pain inputs are integrated, through which, pain signals are forwarded to cortical and sub-cortical brain regions, mainly to somatosensory cortex for the surveillance of pain, and limbic system for the affective components of pain (Bester et al., 2000; Tracey, 2005). Also, the midbrain periaqueductal gray (PAG) and the rostral ventral medulla (RVM) are involved in pain modulation by either inhibiting or facilitating the spinal nociceptive input (Porreca et al, 2002).

1.1.3 Thermal pain

Physiological studies showed that human subjects feel cold pain below 15°C and heat pain at above 47°C (Morin and Bushnell, 1998), suggesting that there are cutaneous nociceptors that responding to noxious thermal stimulation. The cell membrane of these primary afferent fibers expresses receptors known as transient receptor potential (TRP) ion channels that are specified for converting thermal and chemical stimuli into electrical signals (Pertovaara, 2013).

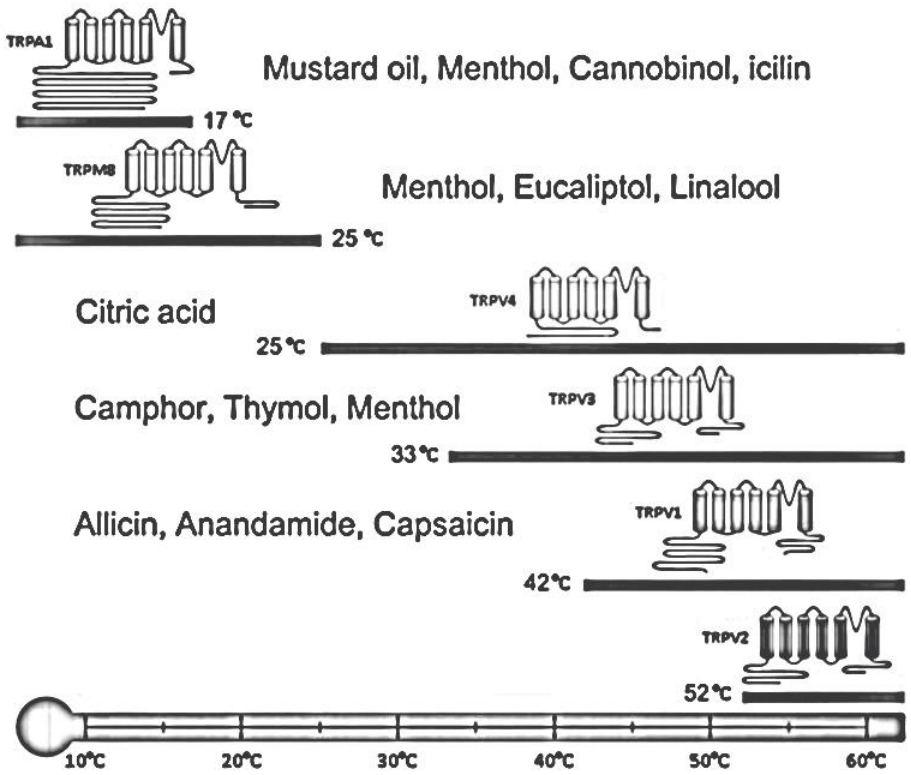

Fig. 1 Thermal TRP channels, their temperature responding ranges, and activation profile by chemicals.

Mammalian TRP channels that are expressed in sensory nerve endings are characterized by their distinctive temperature-dependent activation patterns (Fig. 1). There are two TRP channels expressed in sensory neurons for the perception of cold, namely TRPA1 and TRPM8. TRPA1 is activated by noxious cold temperatures below 17°C, while TRPM8 responds to gentle cooling with an activation threshold of about 25°C. TRPV4 and TRPV3 are activated by warmer temperatures, above 25°C and 33°C respectively. TRPV1 and TRPV2 are activated by noxious heat with respective thresholds of 42°C and 52°C (Belvisi et al., 2011). All TRP channels can also be activated by various chemicals present in the environment (Fig.1).

Of the TRP channels expressed on nociceptive nerve fibers, the best known are TRPA1 and TRPV1. TRPA1 is activated by various irritant compounds such as mustard oil and icillin (Fig. 1), as well as noxious mechanical stimulation (Patapoutian et al., 2009). TRPA1 deletion in mice resulted in deficits in behavioral responses to noxious cold, assessed using the cold plate (0°C), and acetone tests (Kwan et al., 2006). TRPV1 is activated by painful heat and chemicals such as capsaicin (Fig. 1). However, studies have shown that the cold activated current was blocked by high dose of TRPV1 antagonist capsazepine (Foulkes and Wood, 2007), indicating there is also a potential role played by TRPV1 in cold pain transduction.

Unlike TRPA1 and TRPV1, which are predominately expressed in nociceptive nerve endings, TRPM8 is expressed in 5-10% of the DRG neurons with both non-nociceptive and nociceptive nerve fibers (Mckemy et al., 2002). Genomic knockout of TRPM8 produced a marked reduction of the response to topical acetone, which is a cold stimulus in mice without affecting noxious cold senses (Colburn et al., 2007). Neurons that responding to innocuous cool and noxious cold are found in lamina I of the spinal cord and trigeminal dorsal horn and have been shown to project to the thalamus in the brain (Dostrovsky and Craig, 1996). Stimulation of TRPM8 might be able to modulate pain since it could potentially activate a subpopulation of the presumed spinal pain–relay neurons (Pertovaara, 2013).

1.2 CHRONIC NEUROPATHIC AND RHEUMATOID ARTHRITIS PAIN

1.2.1 Neuropathic pain

In the European Union the prevalence of chronic pain is around 20% in adults and imposes a huge burden on society (Breivik et al., 2006). Neuropathic pain accounts for most of the worst clinical chronic pain situations and disabilities caused by chronic neuropathic pain significantly decreases the quality of life not only in the patients themselves but also in their care-giving family members (Ebrahimzadeh et al., 2013). Neuropathic pain can be classified into peripheral or central neuropathic pain depending on the location of primary lesion (Jensen et al., 2001) or categorized according to the etiological diagnosis, for example painful diabetic neuropathy, trigeminal neuralgia, postherpetic neuralgia, and posttraumatic neuralgia.

The major symptoms of neuropathic pain are spontaneous pain, allodynia, hyperalgesia and the loss of sensory functions (Jensen et al., 2001; Baron et al, 2010). Allodynia is abnormal pain perception by stimulations that generally do not cause any tissue damage. Mechanical allodynia is thought to be mediated by the low threshold Aβ fibers. Therefore, gentle brushing of the skin can evoke intense pain sensations in neuropathic pain patients (Jensen et al., 2001). In contrary, hyperalgesia is a term used to describe the exaggerated

response to a noxious stimulus. Spontaneous pain in neuropathic pain is stimulus-independent, and can be either continuous or paroxysmal. It is usually described as shooting, electric shock-like, burning or stabbing. Partial or complete loss of afferent sensory function is another essential consequence in neuropathic pain conditions, leading to sensory deficit in these patients (Jensen et al., 2001).

The mechanisms of neuropathic pain have been extensively studied. It is generally believed that complex changes in the peripheral and central nervous system that related to sensory pathways are initiated following peripheral injury. Damaged afferents become the source of abnormal neuronal activation, arising either from the injured axon or the cell body in the DRG (Wall and Devor, 1983). The ongoing inputs from these afferents subsequently induce hypersensitivity in dorsal horn neurons, a process sometimes known as central sensitization (Woolf, 1983). Activation of trans-membrane G coupled N-Methyl-D-aspartate (NMDA) receptors in the spinal cord is one of the key component in central sensitization (Woolf, 1983). Another central change that is known to occur in neuropathic pain states is the deficiency in inhibitory mechanisms, in particular, loss of gamma-aminobutyric acid (GABA)-ergic and glycinergic neuronal transmissions (Castro-Lopes et al., 1993). Finally, nerve injury produced marked plasticity alterations in the DRG and central nervous system (CNS), including expression changes of ion channels and neuropeptides in sensory neurons, which are related to the development of neuropathic pain (Hökfelt et al., 1994).

1.2.2 Pain in Rheumatoid Arthritis

Chronic pain is one of major symptoms in rheumatoid arthritis (RA), and the primary reason for RA patients to seek medical care. There are several factors impacting RA pain in human. For instance, women usually have higher pain ratings than men (Wolfe and Michaud, 2007). Furthermore, RA pain is most common between 50 and 62 years of age, which may be related to changes in physical activity (Wolfe and Michaud, 2007). In the joints all structures with the exception of cartilage are innervated by nociceptors. In RA, the auto-immune reaction elicits physical and biochemical changes leading to local secretion of inflammatory mediators including prostaglandins and cytokines. These inflammatory mediators sensitize nociceptors in the joints, resulting in the perception of evoked pain during movement and spontaneous pain during the resting state (Bas et al., 2012). In addition to acute pain experienced during disease flares, more than 40% of the RA patients also develop chronic pain within 5 years after the disease onset (Andersson et al., 2013). The prevalence of spread pain symptoms such as fibromyalgia is also higher among the patients with RA than in general population (Lee, 2013).

Clinical experience suggests that in RA patients there is poor correlation of pain symptoms and the peripheral inflammation. The treatment of inflammatory incidences in disease flares of RA has been markedly improved since the introduction of biologic drugs. These therapies can successfully halt the progression of inflammation by blocking the effect of cytokines, T cells and B cells. However, despite the improvements in disease control, pain, particularly chronic pain, is still a problem in many RA patients (Wolfe and Michaud, 2007; Andersson et al. 2013).

1.2.3 Animal models of chronic pain

The application of clinically relevant animal models is of paramount importance in studies of the mechanisms and treatments of chronic pain. In neuropathic pain research, the development of the chronic nerve constriction model by Bennett and Xie (1988) was

an important milestone as they showed that it is possible to produce a partial injury to the sciatic nerve, enabling the observed behaviors of animals to be similar as human conditions of hyperalgesia and allodynia. Since then, a large number of models has been developed using a variety of methods to induce injury in different nerves. These models have greatly facilitated the experimental research on neuropathic pain (Xu and Wiesenfeld-Hallin, 2003).

Our laboratory has been using a photochemical technique to produce ischemic injury to the spinal cord and peripheral nerves. This method involves intravascular interaction between a photosensitizing dye and a laser beam at the appropriate wavelength, leading to the generation of singlet oxygen radicals at the endothelial cells of capillaries and subsequent platelet aggregation within the blood vessels in the irradiated nervous tissues (Watson et al., 1986; Kupers et al., 1998). Our laboratory developed one of the first spinal cord injury (SCI) pain models using this method (Xu et al., 1992). Thus, after spinal ischemic injury rats developed marked pain-like behaviors to mechanical and cold stimulation in the dermatomes corresponding to injured spinal segments in a manner that is similar to SCI patients (Hao et al. 1991, 1992, Xu et al. 1992, 1994). This model has been used to test the efficacy of a large number of anagesics against central pain. The photochemical technique has also been used in producing partial sciatic nerve injury in rats and mice (Kupers et al. 1998; Hao et al. 2002) and infraorbital nerve injury in rats (Eriksson et al., 2005). Some of these models are used in the present work to test the effect of analgesics.

Several rodent models of RA have been developed based on pathophysiological mechanisms of the disease. One of the early models is the adjuvant arthritis (AA) model in rats which involves injecting Freund's complete adjuvant into susceptible strains of rats resulting in a T cell-mediated autoimmune arthritis. Another widely used model is the collagen-induced arthritis (CIA) model in which autoimmune arthritis is produced in some strains of mice by immunization with an emulsion of complete Freund's adjuvant and type II collagen (CII). In the present thesis, we have utilized the collagen antibody-induced arthritis (CAIA) model, which is a novel mouse model of RA, based on the injection of a cocktail of monoclonal antibodies targeted against type II collagen following by lipopolysaccharide (LPS) immunization (Nandakumar and Holmdahl, 2007). In this model, the local joint pathology resembles that observed in RA patients, and there is a development of robust pain-like behaviors (Bas et al., 2012). Important advantages of the CAIA model include: (i) good overall health for the affected mice, (ii) shortened disease duration as it does not depend on breakage of tolerance and bypasses the natural development of anti-collagen antibodies and (iii) the fact that it can be generated in many strains of mice that are resistant to CIA (Nandakumar and Holmdahl, 2007).

1.3 SEX DIFFERENCES

1.3.1 Sex differences in pain

As an important modulator for the perception of pain, sex has been increasingly recognized and studied by the international pain research community. It has been recognized that there is a sex difference in pain sensitivity. Females generally have lower pain threshold and tolerance than males (Berkley, 1997; Fillingim et al, 1999; Mogil, 2000; Barrett et al, 2002; Wiesenfeld-Hallin, 2005; Greenspan et al, 2007). In addition, women also experience more variable pain episodes, which usually last longer than in men with similar conditions (Berkley, 1997; Hurley and Adams, 2008). These

differences, however, are small, exist only for certain forms of stimulation modalities and are affected by many factors such as the presence of disease and experimental setting (Berkley 1997). A large number of chronic pain conditions, e.g. migraine, temporomandibular disorder (TMD), RA, irritable bowel syndrome (IBS), and fibromyalgia are more common in women (Ektor-Andersen et al, 1993; Whitacre, 2001; Craft et al, 2004; Holdcroft and Berkley, 2005; Greenspan et al, 2007). The response to analgesics has also been reported to be sex dependent. For example, morphine appears to have a stronger analgesic effect in males than in females (Berkley 1997; Mogil, 2000).

Estrogen exerts its effects via interaction with receptors which belong to a super family of nuclear receptors. Activation of estrogen receptors (ERs) in the nucleus induces an estrogen response element, leading to modulation of transcription of estrogen-regulated genes. Two forms of ERs have been identified, namely ERα and ERβ (Gruber et al, 2002; Koehler et al, 2005). Previous studies have shown that both ERα and ERβ are presented in the CNS and also expressed in DRG neurons (Papka and Storey-Workley, 2002), suggesting their potential role in sensory modulation. In our lab, we have shown that either knocking out ERα or ERβ can render female mice responding to pain more like males with increased basal pain threshold and a higher resistance to carrageenan induced inflammatory pain (Li et al., 2009).

1.3.2 Sex differences in Rheumatoid Arthritis

Basic immune responses differ between females and males, i.e. following immunization, female mice produce more antibody and show more vigorous T cell activation than male mice. Women have higher absolute numbers of cluster of differentiation (CD) 4^+ lymphocytes in comparison to men, which may contribute to their increased immune responses (Whitacre, 2001). One of the important clinical features of RA is the higher incidence of occurrence in women. A large number of epidemiological studies have shown that, in general, the sex ratio (women vs. men) of RA is typically at about 3:1, and women usually have higher pain ratings during the disease than men (Whitacre, 2001; Wolfe and Michaud, 2007).

Changes in levels of circulating gonadal hormones have been implicated to be one of the main causes for the overrepresentation of women in RA and RA associated chronic pain. Pregnancy appears to protect against the development of RA and ameliorate RA symptoms with most profound effect during the third trimester, when estrogen and progesterone concentrations reach their peak (Whitacre, 2001), which is reversed shortly post partum. In contrast, the incidence of RA in women peaks after menopause where the reduction in estrogen level has been shown to facilitate ongoing pain in RA and also in TMD and migraine (LeResche et al, 1997; Brandes, 2006). Interestingly, men who develop RA have significantly lowered testosterone levels compared with healthy subjects (Whitacre, 2001), indicating that the sex hormones estrogen, progesterone and testosterone can modulate RA disease activity.

In animal models of RA the role of sex has been unclear. Experimental studies of CIA have shown that in some mouse strains there was a reversed sex difference compared with the clinical situation in that male mice displayed a higher incidence of arthritis than females (Holmdahl et al., 1989; Jansson et al., 1994). Ovariectomy (OVX) or blockade of estrogen receptors in these females appears to increase the incidence and severity of arthritis whereas treatment with estradiol suppresses symptoms in ovarietomized female mice (Jansson et al., 1994). However, whether such pattern of sex difference is also true

for other preclinical models of RA generated in other strains, and with regard to RA induced arthritic pain, remains to be further examined.

1.4 ANALGESICS

1.4.1 Analgesics in neuropathic pain

Neuropathic pain is difficult to treat using available pharmacological agents. The non-steroidal anti-inflammatory drugs (NSAIDs), which are the most commonly used pain medicines worldwide, have no effect in neuropathic pain. Opiates, the ultimate class of strong analgesic, have at best ambiguous effects against neuropathic pain in the majority of patients (Arnér et al., 1998). Opiates also produce significant side effects, including respiratory depression, constipation and long term administration is associated with tolerance, dependence and abuse.

The first line of analgesics prescribed today to treat neuropathic pain is gabapentin and pregabalin (Attal et al. 2010). Gabapentin and pregabalin are structurally related to GABA, but their effects appear not to be mediated by the GABAergic system. Instead, they interact with the α2δ subunit of the voltage-dependent L-type calcium channel in the CNS, suppressing neuronal excitability and decreasing the release of neurotransmitters. The clinical indication for gabapentin and/or pregabalin in neuropathic pain include diabetic neuropathy, post-herpetic neuralgia, central pain and fibromyalgia, but evidence indicate that these drugs can only achieve partial relief of pain in patients (Serpell, 2002; Gordh et al., 2008).

Another class of analgesics that may be potentially used in neuropathic pain are antagonists of the NMDA receptors, particularly those of non-competitive and low affinity nature such as dextromethorphan (Hao and Xu, 1996). However, despite strong pre-clinical results, there is still a lack of convincing clinical evidence for analgesic effect of this type of drugs in neuropathic pain.

1.4.2 Analgesics in arthritic pain

Disease-modifying anti-rheumatic drugs (DMARDs) such as methotrexate are known to reduce acute symptoms in RA including pain, but their efficacy against the development of chronic pain is less clear (Taylor et al., 2010; Andersson et al., 2013; Steiman et al., 2013). In early RA patients, treatments with NSAIDs, acetaminophen, biological drugs or sometimes weak opioids have been shown to be effective in reducing acute inflammatory pain (Strand et al., 2012, Lee et al. 2013). However, these drugs appear to have limited effects against chronic pain in the absence of joint inflammation (Whittle et al., 2012; Andersson et al., 2013; Lee et al. 2013). Long term application of these drugs are however limited by side effects and in the case of opioids, the development of tolerance (Lang et al., 2010).

1.4.3 Sinomenine

Botanicals, compounds extracted from plants, have contributed significantly to our arsenal of pharmacological treatment of diseases. The discovery of artemisinin for treating malaria is probably the best example of a successful development of a novel drug from Chinese herbal medicine, a feat that will likely be repeated many times in the future.

Sinomenine is a morphinan derivative alkaloid that is structurally similar to dextromethorphan (Fig. 2), found in the root of the climbing plant *Sinomenium Acutum* which is native to Japan and China. Sinomenine has long been used in East Asia as a remedy for disease conditions similar to rheumatism as recorded in the 16th century book, Bencao Gangmu (Compendium of Materia Medica). It is still clinically used in China and Japan for conditions such as RA, arrhythmia and neuralgia (Yamasaki, 1976). In mice, treatment with sinomenine decreased the incidence and severity of arthritis in the CIA model (Huang et al., 2007). Compared with NSAIDs, sinomenine was more effective in the reduction of morning stiffness, painful joints and erythrocyte sedimentation rate in RA patients (Xu et al., 2008). Clinical studies also demonstrated that sinomenine may be effective in relieving pain in RA and some types of neuralgia, such as sciatic neuritis, lumbalgia and muscular rheumatism (Yamasaki, 1976).

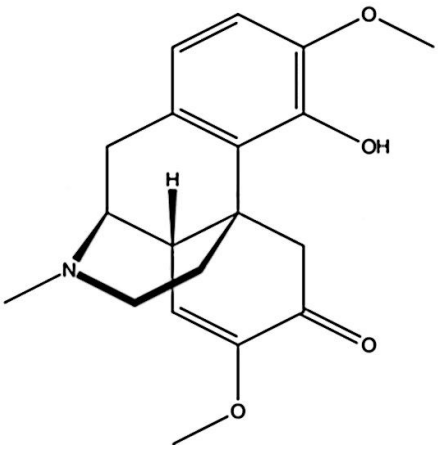

Fig. 2 Chemical Structure of Sinomenine

Receptor binding studies have shown that sinomenine is able to activate the opioid μ receptor *in vivo* at high concentrations and long-term pretreatment with sinmenine may delay the onset of morphine analgesic tolerance (Wang et al., 2008). In addition, sinomenine can downregulate the elevated levels of cyclic adenosine monophosphate (cAMP), 5-hydroxytryptamine (5-HT), noradrenaline, dopamine, and neuronal nitric oxide synthase (nNOS) in the cerebral cortex (Wang et al., 2002 and 2003), to maintain a normal state in morphine-dependent, naloxone-precipitated withdraw rats. Furthermore, SIN exhibited anxiolytic-like effect that resembling the effect of the partial 5-HT1A agonist Gepirone (Chen et al., 2005).

2. AIMS OF THE THESIS

The general aims of the thesis are to use experimental methods to study the mechanisms and treatments of chronic pain.

In particular:

1. To develop a method to quantitatively examine pain-like responses to thermal (heat and cold) stimulation in spinally injured rats.

2. To validate CAIA as a model of arthritic pain.

3. To study the potential analgesic of sinomenine in acute nociceptive pain and in chronic neuropathic and arthritic pain models.

4. To study sex difference in the development of arthritis and pain in the CAIA model and to explore the mechanisms of sex differences.

3. MATERIALS AND METHODS

3.1. ANIMALS

All experiments were approved by regional animal research ethics committee and were carried out according to the Ethical Guidelines of IASP. Sprague-Dawley (SD) rats of both sexes (Harlan, Horst, The Netherlands; Möllegård, Denmark), male C57BL/6 mice (Charles River, Sollentuna, Sweden), and CBA mice of both sexes including females which were ovarectomized at 20 days of age at the facility of the animal provider (Harlan, Horst, The Netherlands) were used. Animals were housed 4 per cage for rats and 6 per cage for mice at a constant room temperature of 22°C in a 12:12h light-dark cycle with *ad libitum* access to food and water.

3.2. INFLAMMATORY PAIN MODELS

3.2.1 Collagen antibody induced arthritis model

As described previously (Bas et al., 2012) , CAIA was induced in mice by intravenous (i.v.) injection of anti-CII arthritogenic cocktail (0.15 ml, Chondrex, USA) containing 5 monoclonal antibodies at day 0, followed by intraperitoneal (i.p.) injection of 35 µg LPS (serotype O55:B5; Sigma) in 100 µl of physiologic saline on day 5. Control groups received 100 µl of saline i.v. on day 0. On day 5, the saline control group received i.p. saline, while the LPS control group received i.p. LPS. Inflammation in the joints was evaluated by visual inspection and scored every third day after injection of the antibody cocktail. The scoring was based on the number of inflamed joints in each paw, inflammation being defined by swelling and redness (Nandakumar and Holmdahl, 2007). Briefly, each inflamed toe or knuckle gave one point, an inflamed wrist or ankle gave five points, resulting between 0 and 60 points for each mouse.

3.2.2 Carrageenan-induced inflammation in mice

Mice were anaesthetized with 75 mg/kg ketamine + 1 mg/kg medetomidine in a volume of 1 ml/kg, and λ-carrageenan (Sigma-Aldrich, 20 µl, 2%) was injected subcutaneously (s.c.) into the plantar surface of one hind paw. Mechanical and heat threshold of the inflamed hind paw was tested 24h after the injection.

3.3. NERVE INJURY MODELS

3.3.1 Photochemically induced sciatic nerve injury in mice and rats

Detailed methods for producing sciatic nerve ischemic injury (SNI) have been described previously for rats (Kupers et al., 1998) and mice (Hao et al., 2002). Briefly, animals were anaesthetized by 75 mg/kg ketamine + 1 mg/kg medetomidine and the left sciatic nerve was exposed. After i.v. injection of the photosensitizing dye erythrosine B (Red N°3, Aldrich-Chemie, Steinheim, Germany) at the dose of 32.5 mg/kg, sciatic nerve was irradiated under an argon ion laser (514 nm, 160 mW, Innova model 70, Coherent Laser Product Division, Palo Alto, CA) for 45 s or 2 min for mice or rats, respectively.

3.3.2 Photochemically induced spinal cord injury in rats

Photochemically induced spinal cord ischemic injury method has been described previously (Hao et al., 1992). Briefly, the rats were anaesthetized with 75 mg/kg ketamine + 1 mg/kg medetomidine in 1 ml/kg and one jugular vein was cannulated. A midline incision was made in the skin overlying vertebral segments T12-L1. The animals were positioned beneath the argon laser beam and irradiated for 10 min with the beam directed towards vertebral segment T12 or T13 (spinal segments L3-5). Immediately prior to and 5 min after the start of the irradiation, erythrosin B (Red N°3, Aldrich-Chemie, Steinheim, Germany) dissolved in 0.9% saline was injected i.v. at a dose of 32.5 mg/kg. During irradiation, the body temperature of the rats was maintained at 37-38°C.

3.4. BEHAVIORAL TESTS

3.4.1 Hot plate test in rats

The antinociceptive effect of Sinomenine in normal rats was assessed using a hot plate (IITC, Woodland Hills, CA) which was maintained at 54 °C ± 1 °C. The latency to lick a hind paw was measured with an accuracy of 0.1 s and the cut-off value was set at 30 s to prevent tissue damage. Before testing, the rats were habituated in the testing room for at least 30 min. The rats were trained on the hot plate for 4 days with 2 trials/day to obtain a stable baseline response prior to the experiment.

3.4.2 Tail flick test in mice and rats

In the tail flick test the mice or rats were gently restrained and a radiant heat source (Ugo Basile, Italy) was focused 1 to 2 cm from the tip of the tail. Response latency was automatically recorded. The intensity of the stimulation was adjusted so that the baseline latency was from 4 to 6 s and the cut-off value was 10 s

3.4.3 Paw withdrawal threshold to mechanical stimulation in rats and mice

The withdrawal threshold of the ipsilateral hind paw to mechanical stimulation after sciatic nerve injury in rats was tested using a set of calibrated von Fray hairs (Stoelting, Chicago, IL, USA). Briefly, rats were placed in plastic cages with a metal mesh floor. After habituation for 1 h, the plantar surface of the ipsilateral hind paw was stimulated with increasing force. Each filament was applied 5 times and response threshold was reached when the animal withdrew the paw at least 3 times. The cut-off value was 60 g. The hind paw withdraw threshold to mechanical stimulation in mice with sciatic nerve injury or carrageenan-induced inflammation was tested using the same set of calibrated von Fray hairs (Stoelting, Chicago, IL, USA) in a way similar to that in rats except that the cut-off value was 4 g.

In CAIA mice, control baselines were measured five times every third day before the collagen antibody injection. Animals with baseline threshold below 50% of the average value were excluded from the experiment. Mechanical threshold was tested every third day for 54 days at the same time during the day. For testing of the paw withdrawal threshold mice were randomly placed in plastic cages with a metal mesh floor. After habituation for one hour, the plantar surface of the hind paw was stimulated with a set of calibrated von Frey hairs (Marstock, Denmark), using the up-down method (Chaplan et al., 1994) to calculate the force that caused paw withdrawal in 50% of trials. For testing forepaw withdrawal threshold another set of von Frey hairs (Stoelting, Chicago, IL, USA)

was used. Stimuli were applied 4 to 8 times to the plantar surface of the forepaw with the frequency of 1/s at each force. The stimulus which induced consistent withdrawal (>75% respond rate) was considered as forepaw withdrawal threshold.

3.4.4 Assessment of spread mechanical allodynia in spinally injured rats and CAIA mice

Sensitivity to mechanical stimulation in SCI rats was tested by examining the vocalization thresholds to graded mechanical touch/pressure applied with calibrated von Frey hairs (Stoelting, Chicago, IL, USA). During testing the rats were gently restrained in a standing position and the von Frey hair was pushed onto the skin until the filament became bent. The frequency of the stimulation was about 1/s and 5 to 10 stimuli were applied at each force. The intensity of stimulation which induced consistent vocalization (>75% response rate) was considered as pain threshold. For testing spread mechanical hypersensitivity in CAIA, mice were gently restrained in a standing position. The flanks and upper back were stimulated using the same set of von Frey hairs. Stimuli were applied 5 to 10 times with frequency of 1/s, at each force. The intensity which induced consistent vocalization (>60% respond rate) was considered as vocalization threshold. The cut-off value was 100g on the flanks and back.

3.4.5 Thermal stimulation using a Peltier thermode in SCI rats

The Peltier effect is an end-to-end transfer of heat when electric current is passed in a circuit consisting of two dissimilar semiconductors that result in cooling off one junction while heating up the other. The surface temperature of a Peltier thermode can be maintained or adjusted by varying the current. The development and application of a Peltier thermo stimulator in rats has been described previously (Wilcox et al., 1984). In this study, a fluid cooled, hand held Peltier thermode (active surface: 25 x 50 mm, control resolution: >0.02 °C, calibration uncertainty: +/- 0.2 °C) connected to a Modular Sensory Analyzer (MSA) Thermal Stimulator (Somedic, Sweden) was used for thermal stimulation. The baseline temperature was 32°C and the rate of temperature change was 0.5°C/s. Rats were held gently in a standing position and the thermode was pressed against the shaved flank area. Three heating stimuli were applied at 1 min intervals and the average temperature at which the rats vocalized was taken as heat response threshold with 50°C as cut-off temperature. Similarly, three cooling stimuli were then applied at 1 min intervals and the average temperature at which the rats vocalized was taken as cold response threshold with 6°C as cut-off temperature.

3.4.6 Measurement of cold hypersensitivity using ethyl chloride spray

SCI rats were gently restrained in a standing position and ethyl chloride spray (Rönnings Europa AB, Sweden) was applied to the shaved allodynic flank area. The response was graded with a score of 0 = no observable response; 1 = localized response (skin twitch and contraction), no vocalization; 2 = transient vocalization, moderate avoidance and 3 = sustained vocalization and avoidance. For SNI rats and mice, the immediate response after acetone application on the hind paw was observed and scored for both mice and rats as follows: 0= no responses; 1= startle response without evident paw withdrawal, 2 = withdraw of the stimulated hind paw, 3 = sustained withdraw of the simulated hind paw with flitching or licking.

3.4.7 Test of heat hyperalgesia in mice with carrageenan-induced inflammatory pain

For test of heat hyperalgesia, mice were gently restrained and a radiant heat source (Ugo Basile, Italy) was focused on the plantar surface of the hind paw. The intensity of the stimulation was adjusted so that the baseline latency was from 4 to 6 s and the cut-off value was set at 10 s.

3.4.8 Tactile response test

For examining the response to brush stimuli, the skin on the flanks was briskly stroked with the point of a pencil in a rostral to caudal direction. The response of the animals was graded with a score of 0 = no response, 1 = moderate efforts to avoid the probe but no vocalization, 2 = clear avoiding behavior to the stimulus with transient vocalization, and 3 = vigorous efforts to avoid the stimulus, sustained vocalization in response to the probe.

3.4.9 Motor tests

We have used a combined motor tests of walking in an open field and a righting reflex to detect the potential motor and sedative effect of sinomenine in spinally injured rats which are most prone to motor and sedative effect of drugs (Table 1).

Grade	Description	Score
Walking		
0	Normal walking	0
1	Walks with only mild deficit	5
2	Walks with deficit, hind limb can support weight	15
3	Frequent movement of hind limb, no weight bearing	25
4	Minor movement in hind limb, no weight bearing	40
5	No movement of hind limb, no weight bearing	45
Righting		
0	Normal righting counter to the direction of roll	0
1	Weakened attempt of righting	5
2	Delayed attempt of righting	10
3	No attempt of righting	15

Table 2. Combined motor score of rats

3.5. IMMUNOHISTOCHEMICAL STUDY

Animals were deeply anesthetized with sodium pentobarbital (Mebumal; 50 mg/kg, i.p.) and transcardially perfused with 20 ml warm saline (0.9%, 37°C), followed by 20 ml of warm picric acid-paraformaldehyde (PFA) fixative solution (4% PFA with 0.2% picric acid in 0.16 M phosphate buffer, pH 7.35, 37°C), and then 50 ml of the same fixative at 4°C. The L4 and L5 DRGs as well as the L4 and L5 segments of the spinal cord were dissected out and post fixed in the same fixative for 3h at 4°C, and subsequently transferred to 20% sucrose in phosphate-buffered saline (PBS; pH7.4) containing 0.01% sodium azide (Sigma) and 0,02% bacitracin (sigma) at 4°C for 2 days. Tissues were embedded with OCT compound (Tissue Tek, Miles Laboratories, Elkhart, Ind., USA), frozen and cut in a cryostat (Microm, Heidelberg, Germany) at 12µm (DRGs) or 20µm (spinal cords). For single "TSA plus" staining, mounted sections were dried at RT for 30

min and incubated with primary antibodies against ATF-3 (Santa Cruz, rabbit, catalogue #sc-188,1:4,000), GAP-43 (Chemicon, rabbit, catalogue #AB5220, 1:1,000), Iba-1 (WAKO, rabbit, catalogue #019-19741, 1:2,000), GFAP(DAKO, rabbit, catalogue #Z0334, 1:8,000), galanin (rabbit, 1:4,000; Theodorsson and Rugarn, 2000), SP (rabbit, 1:4,000; Christensson-Nylander et al., 1986), CGRP (rabbit, 1:32,000; Orazzo et al., 1993), CD68 (Abcam rabbit, catalogue #AB125212, 1:2000) overnight at 4°C in a humid chamber. Immunoreactivity was visualized using the tyramide signal amplification system (Perkin Elmer, USA). Briefly, the slides were rinsed with TNT buffer (0.1M Tris–HCl, pH 7.4; 0.15 M NaCl; 0.05% Tween 20) for 15 min, blocked with TNB buffer (0.1M Tris–HCl; pH 7.4; 0.15M NaCl; 0.5% blocking reagent (Perkin Elmer, Boston, MA) for 30 min, followed by a 30-min incubation with secondary antibody diluted in TNB buffer. After a quick wash (15 min) in TNT buffer, all sections were exposed to biotinyl tyramide-fluorescein (1:100) diluted in amplification diluent for 10 to 15 min (all steps at RT). For double staining, CD68 was stained using tyramide signal amplification, while Iba-1 primary antibody was incubated with secondary antibody (Invitrogen, goat anti rabbit, catalogue #A11037, 1:200) at the following day. DRG Sections were counterstained for 15 min with 0.001% (w/v) propidium iodide (PI, Sigma) in PBS, and all slides were coverslipped with anti-fading mounting medium (DABCOTM, Sigma).

Images were captured by a 710 LSM system (Zeiss, Jena, Germany) and operated by LSM ZEN2009 software (Zeiss). Multi-panel figures were assembled in Adobe Photoshop CS5 software (Adobe Systems Inc., San Jose, CA). Quantification in DRG staining was done by using the percentage of positive neurons over all neurons (for galanin, ATF-3, GAP-43, SP and CGRP), and the number of positive cells colocalized with PI in a randomly selected region (232μm × 232μm, for Iba-1). In spinal cord stainings, signal intensity of markers expressed in the dorsal horn region was measured and normalized with background value.

3.6. QPCR STUDY

On days 15 and 54 following the induction of CAIA, lumbar spinal cords and L4 and L5 DRGs were dissected and immediately frozen at -80°C. Before qPCR analysis, mRNA was extracted using TRIzol (Invitrogen), and complementary DNA was produced. As to determine relative mRNA levels, quantitative real-time PCR was performed with TaqMan Gene Expression Assays (Applied Biosystems), using the GeneAmp 7500 Fast Sequence Detection system (Applied Biosystems). Pre-developed specific primers were used to detect HPRT1 (reference gene, TaqMan, Mm00446968_m1), galanin (TaqMan, Mm01236508_m1), ATF-3 (TaqMan, Mm00476032_m1) and GAP-43 (TaqMan, Mm00500404_m1) signals. Sample threshold cycle values in standard curve samples (mouse RAW 264.7 cells stimulated with LPS for 4h and standard spinal cord or DRG tissues from CAIA mice) were used to calculate the cDNA concentration equivalents in the DRG and spinal cord samples, and then the data were normalized to HPRT gene expression to obtain relative concentrations and presented as relative expression units.

3.7. DRUGS

For preparation of injecting solutions, sinomenine (standard substance was obtained from The National Institute for Food and Drug Control, Beijing, China) was firstly dissolved with DMSO (Sigma-Aldrich), then mixed with Cremophor EL oil (Sigma-Aldrich) and saline by a vortex mixer (Bibby Scientific, UK) using the volume rate of 1:4:5. Any further dilution was made with saline. The opioid receptor antagonist naloxone was obtained from Tocris (Bristol, UK) and dissolved in saline. For single dose application,

sinomenine was administered i.p., s.c., or p.o. in rats and mice. To perform oral administration, the rat/mouse was held in an upright standing position and a bulb tipped gastric gavage needle was used to deliver the sinomenine solution into the stomach by the attached syringe. For chronic administration, sinomenine was administered twice daily for 5 days at 10:00 h and 16:00 h.

3.8.STATISTICS

Statistics were performed using Statview software (SAS Institute Inc., USA). The experiments were conducted blindly. Data were presented as mean ± error of the mean (SEM) or median ± median absolute deviation (MAD), and were analyzed with analysis of variance (ANOVA) with/without repeated measurements or the Kruskal–Wallis test, followed by Bonferroni/Dunn's test, Fisher's Protected Least Significant Difference (PLSD) test, Dunnett's test, Wilcoxon signed rank test, and paired t-test. For unpaired comparisons, the Mann-Whitney U test and unpaired t test were used. Simple linear regression analysis was performed and the significance of correlation was tested by ANOVA. For all the statistics, $p<0.05$ was considered as significant.

4. RESULTS

4.1. QUANTITATIVE TEST OF RESPONSES TO THERMAL STIMULATION IN SPINALLY INJURED RATS (PAPER I)

4.1.1. The development of mechanical and cold hypersensitivity in spinally injured rats

The vocalization threshold to stimulation with von Frey hairs in normal rats was 60 -100g. Photochemically induced SCI produced marked mechanical hypersensitivity in the majority of rats starting at day 1 with vocalization threshold of 2-6 g, which was maintained for at least 70 days (Fig. 2a, Paper I). Mechanical allodynia was present on the flank and lower back areas corresponding to the dermatome of injured spinal segments. In the same area, cold stimulation with ethyl chloride spray triggered pain-like response in SCI allodynic, but not normal rats (Fig. 2b, Paper I). Such cold allodynia could be detected 1 day after injury and was maintained for the 70 days of observation period (Fig. 2b, Paper I). A sub-population of rats (10-20 %), however, did not exhibit mechanical hypersensitivity after SCI (non-allodynic rats).

4.1.2. Quantitative thermal testing in spinally injured rats

Normal rats had no aversive response to cooling by a Peltier thermode from 32°C to 6°C on the flank area. The vocalization threshold of SCI rats was increased significantly from day 1 (Fig. 3a, Paper I), and lasted for at least 70 days (Fig. 3a, Paper I). The cold hypersensitivity was prominent in the first two weeks and almost all rats demonstrated a cold pain threshold above 17°C immediately after injury. Towards the end of the 10 week observation, around 50 % of rats still had consistent vocalization threshold above 17°C.

The majority of normal rats did not exhibit pain-like response to warm/heat stimulation up to 50°C, with few responses between 47-50°C. The heat response temperature was not significantly decreased in SCI rats throughout the whole observation period (Fig. 3b, Paper I).

4.1.3. Correlation analysis of cold response temperature with cold scores and mechanical response threshold

Using simple linear regression we found that in the same allodynic rats the cold response temperature was highly correlated with the cold response score in individual rats (r^2 =0.612, $p<0.01$) (Fig 4a, Paper I). Furthermore, there was also a significant, albeit less robust, correlation between vocalization threshold to mechanical stimulation and cold response temperature (r^2 =0.231, $p<0.01$) in these rats (Fig. 4b, Paper I).

4.1.4. Cold responses in non-allodynic spinally injured rats

A sub-population of rats did not develop mechanical allodynia after spinal cord injury (non-allodynic rats with mechanical threshold >15g). No significant increase in cold response to ethyl chloride was observed in non-allodynic rats compared with controls (Fig. 5a, Paper I). However, when tested with the Peltier thermode and using response temperature as endpoint, we observed that the non-allodynic rats were hypersensitive to

cold and they differed significantly from both the control group and allodynic group (Fig. 5b, Paper I).

4.2. WIDE-SPECTRUM ANALGESIC EFFECT OF SINOMENINE IN RODENTS AFTER INFLAMMATION AND NERVE INJURY (PAPER II)

4.2.1. Antinociceptive effect of sinomenine in hot plate and tail flick tests

Systemic sinomenine produced antinociception in the hot plate and tail flick tests in male rats (Paper II, Fig.1A, B) at 40 mg/kg, but not at lower doses (10 or 20 mg/kg). The effect was significant at 30 min in the hot plate test (Paper II, Fig.1A) and at 30, 60 and 90 min in the tail flick test (Paper II, Fig.1B). At 10-40 mg/kg sinomenine did not produce any observable side effect such as sedation, allergy or motor impairments. At 80 mg/kg, sinomenine had a moderate sedative effect. Antinociception was also seen in mice at 60 min following 80 mg/kg i.p. sinomenine, but not at lower doses (20 or 40 mg/kg) in the tail flick test (Paper II, Fig. 1C). Sinomenine at 80 mg/kg i.p. did not produce any observable side effects in mice. Vehicle had no effect.

4.2.2. The effect of sinomenine on carrageenan-induced mechanical and heat hypersensitivity

Carrageenan injected s.c. into the plantar surface of hind paws of male mice induced mechanical and heat hypersensitivity at 24 h post injection, which were significantly reduced by sinomenine administered orally (p.o.) in mice at 80 mg/kg (but not at lower doses) for up to 120-180 min (Paper II, Fig. 2A, B). Sinomenine at 80 mg/kg p.o. did not produce any observable side effects in mice.

4.2.3. The effect of sinomenine on mechanical and cold hypersensitivity after peripheral nerve injury in mice and rats

The effect of sinomenine was tested 2 weeks after sciatic nerve injury, when the animals exhibited mechanical and cold hypersensitivity of the hind paws. I.p. sinomenine at 40 mg/kg reversed mechanical and cold hypersensitivity in rats (Paper II, Fig. 3A, B). Mechanical and cold hypersensitivity was also present in mice after sciatic nerve injury. I.p or p.o. sinomenine at 40 or 80 mg/kg dose dependently reduced mechanical hypersensitivity in nerve injured mice (Paper II, Figs. 4A, 5A). Interestingly, sinomenine did not reduce cold hypersensitivity following either i.p. or p.o. administration in mice (Paper II, Fig. 4B, 5B). The effect of 80 mg/kg i.p. sinomenine on mechanical hypersensitivity in nerve injured mice was not reversed by i.p. naxolone (1 mg/kg, 60 min after sinomenine) (Paper II, Fig. 6).

4.2.4. The effect of sinomenine on mechanical and cold hypersensitivity in spinally injured rats

The pharmacological experiments were conducted in female SD rats 4-5 weeks after the induction of spinal cord injury when the animals exhibited hypersensitivity to innocuous mechanical and cold stimulation at the flank area at or just rostral to the dermatome of the injured spinal segments (Xu et al., 1992). I.p. sinomenine at 40 mg/kg, but not lower doses or vehicle, significantly decreased mechanical and cold allodynia for up to 240 min (Paper II, Fig. 7 A, B) without producing any observable side effects.

4.3. REPEATED SINOMENINE ADMINISTRATION ALLEVIATES CHRONIC NEUROPATHIC PAIN-LIKE BEHAVIORS IN RODENTS WITHOUT PRODUCING TOLERANCE (PAPER III)

4.3.1. Effect of repeated administration of sinomenine on pain-like behaviors in spinally injured rats

Sinomenine dose-dependently suppressed hypersensitivity to mechanical (Paper III, Fig 1) and cold (Paper III, Fig 3) stimulation in rats after spinal cord injury. Saline had no effect on either mechanical (Paper III, Fig 1A, 2A) or cold (Paper III, Fig 3A) responses. A single dose of i.p. sinomenine at 10 or 20 mg/kg had no effect on responses to mechanical or cold stimulation in SCI rats (Paper III, Fig 1-3) as previously reported (Paper I).

In contrast, repeated administration of 10 mg/kg sinomenine twice per day elevated vocalization threshold to mechanical stimulations and reduced response score to brushing from day 2 to day 5 of treatment (Papper III, Fig 1B, 2B). However, repeated administration of 10 mg/kg sinomenine had no effect on hypersensitivity to cold (Paper III, Fig 3B). Repeated administration of sinomenine at 20 mg/kg reduced mechanical hypersensitivity to stimulation with von Frey hairs and brushing from day 2 to day 5 (Paper III, Fig 1C, 2C). Furthermore, pre-drug response threshold to von Frey hairs was significantly elevated from day 2 of sinomenine treatment and the threshold remained significantly elevated compared to day 1 for at least 4 days after the cessation of drug application (Paper III, Fig 1C). The pretreatment response score to brushing was also significantly decreased from day 4 to day 6 after the start of drug treatment (Paper III, Fig 2C). However, 20mg/kg sinomenine did not alleviate allodynia to cooling (Paper III, Fig 3C).

Sinomenine administered 2/day at 40 mg/kg effectively reduced mechanical hypersensitivity. Baseline thresholds to stimulation with von Frey hairs was significantly increased from day 2 of treatment and lasted until day 9, 4 days after the last administration of sinomenine (Paper III, Fig 1D). The response threshold returned to pre-drug baseline level on day 12 (Paper III, Fig 1D). Hypersensitivity to brushing was also reversed on days 2, 4 and 5 following repeated Sinomenine (Paper III, Fig 2D). The threshold temperature for cold stimulation was significantly decreased (indicating a decrease in cold hypersensitivity) 2h after sinomenine during the first two days (Paper III, Fig 3D). The pre-drug cold response temperature was significantly reduced from baseline level from day 2 to day 9 (Paper III, Fig 3D), again suggesting a sustained reduction in cold hypersensitivity.

4.3.2. Effect of repeated sinomenine on neuropathic pain-like behaviors in mice following sciatic nerve injury

Saline had no effect on paw withdrawal threshold (Paper III, Fig 4A) or cold sensitivity (Paper III, Fig 4C). Sinomenine at 80 mg/kg administered p.o. twice a day for 5 days produced significantly increased paw withdrawal threshold on days 1 to 5 (Paper III, Fig. 4B). There was also a significant and persistent elevation in pre-drug baseline response threshold to simulation with von Frey hairs from day 2 and was maintained for 7 days after the termination of drug treatment (Paper III, Fig. 4B). Sinomenine also significantly reduced mechanical and cold post-drug responses, in comparison with the pre-drug thresholds (Paper III, Fig 4B, D).

4.4. SINOMENINE ALLEVIATES MECHANICAL HYPERSENSITIVITY IN MICE WITH EXPERIMENTALLY-INDUCED RHEUMATOID ARTHRITIS (CAIA) (PAPER IV)

4.4.1. The dose-dependent effect of sinomenine against mechanical hypersensitivity of the hind paw in mice with CAIA

During the inflammatory phase of CAIA (days 11-19 after CII antibody injection), a single dose of 40 and 80 mg/kg s.c. Sinomenine dose-dependently reduced mechanical hypersensitivity in the hind paws (Paper IV, Fig. 1A). In the post-inflammatory phase during days 35-54 post CII antibody, sinomenine also had a similar effect as during peak inflammation (Paper IV, Fig. 1B). No side effects such as sedation or motor impairments were observed following sinomenine administration.

4.4.2. The dose-dependent effect of sinomenine against spread mechanical hypersensitivity

Mice subjected to CAIA developed, in addition to localized mechanical hypersensitivity of the paws, a spread mechanical hypersensitivity primarily at the neck and flanks (Paper V). A single dose of 40 or 80 mg/kg sinomenine also significantly alleviated the spread mechanical hypersensitivity during both the inflammatory and post-inflammatory phases of CAIA (Paper IV, Fig 2A, B).

4.4.3. Effect of repeated administration of sinomenine

Repeated injection of 80 mg/kg sinomenine 2 times/day for 5 days during days 11-15 post CII antibody administration (inflammatory phase), had no effect on the arthritic scores in mice with CAIA in comparison to saline treated animals (Paper IV, Fig. 3A). Sinomenine administered 2/day for 5 days during the peak of inflammation significantly alleviated the mechanical hypersensitivities in the hind paws (Paper IV, Fig. 3B) and in the neck/flank region (Paper IV, Fig. 3C). Baseline mechanical hypersensitivity was significantly increased from the second day after the start of repeated sinomenine treatment, and remained significantly elevated for 3 days after the cessation of sinomenine treatment (Paper IV, Fig. 3B, C). During the post inflammatory phase, repeated sinomenine administration (at days 49-53 post CII antibody administration, 80 mg/kg, 2/day) significantly alleviated mechanical hypersensitivity both of the hind paws and on the neck/back regions (Paper IV, Fig. 3A, B). Baseline mechanical hypersensitivity was significantly increased from the second day after the onset of repeated sinomenine treatment for the hind paw, but only on day 5 for the spread hypersensitivity (Paper IV, Fig. 3B, C). The effect persisted for at least one day after the cessation of sinomenine treatment as the experiments were terminated on day 54 according to a pre-determined schedule (Paper IV, Fig. 3B, C). No side effects were observed during repeated sinomenine treatments during both the inflammatory and post-inflammatory phases.

4.5. SEX DIFFERENCES IN THE DEVELOPMENT OF ARTHRITIS AND PAIN IN MICE WITH COLLAGEN ANTIBODY-INDUCED ARTHRITIS (PAPER V)

4.5.1 Joint inflammation and effect of ovariectomy after CAIA

Both male and female CBA mice developed joint inflammation (measured with the Arthritis Score) after the induction of CAIA (Paper V, Fig. 1A). Female mice had significantly higher arthritis score than males for forepaws and hind paws over the entire observation period. The female mice had both higher peak and longer duration of joint inflammation as judged by the arthritis scores (Paper V, Fig. 1A). The extent of joint inflammation was also significantly more severe in the forepaws compared to the hind paws, particularly for the male mice (Paper V, Fig. 1A, B). Ovariectomized (OVXd) female mice resembled males in the development of joint inflammation following CAIA (Paper V, Fig. 1C). There were significant overall differences between males and females and between females and OVXd females, but not between males and OVXd females as analyzed using area under the curve (Paper V, Fig. 1C, D).

4.5.2 Development of mechanical hypersensitivity after CAIA and the effects of ovariectomy

Both male and female CBA mice developed mechanical hypersensitivities in hind paws after induction of CAIA, not seen in the saline control groups (Paper V, Fig 2A). Female mice had a significantly lower response threshold to mechanical stimulation than males during the whole 54 day observation period (Paper V, Fig. 2A, B). OVXd female mice also developed significant, mechanical hypersensitivity in the hind paws following CAIA, but not after saline treatment (Paper V, Fig. 2A). The magnitude of mechanical hypersensitivity in CAIA-treated OVXd females was between female mice and male mice, so that there were no significant overall differences between OVXd females and females or between OVXd females and males (Paper V, Fig. 2A, B). Mechanical hypersensitivity was also detected in the forepaws (Paper 5, Fig 2C, D) and in the neck and upper back region as illustrated in both male and female mice after CAIA (Paper V, Fig 2E, F). There were also significant sex differences in spread mechanical hypersensitivity between male and female mice (Paper V, Fig 2E, F).

4.5.3 Expression of Substance P and CGRP in lumbar DRGs and spinal cord dorsal horn

For substance P (SP) and calcitonin gene related peptide (CGRP), the expressing profiles were similar among male and female as well as OVXd mice in the DRG of saline treated groups (around 15 % and 35 % positive neuronal profile in the DRG for SP and CGRP respectively, Paper V, Fig 3A, B, C). The level of SP or CGRP expression were not altered in the DRGs in the CAIA groups on day 15 or day 54 after induction of inflammation (Paper V, Fig 3A, B). The level of SP or CGRP expression was also similar in the dorsal horn among three groups of mice studied and, again, CAIA did not alter the expression pattern of SP or CGRP in the dorsal horn (Paper V, Fig 4A, B, C).

4.5.4 The expression of ATF-3 and GAP-43 in lumbar DRGs

The qPCR analysis revealed equal increases in activating transcription factor 3 (ATF-3) mRNA levels in male and female DRGs 15 days (inflammatory phase), but not 54 days (post-inflammatory phase), after CAIA-induction (Paper V, Fig 5A). The number of

ATF-3 positive DRG neuron profiles (NPs) was significantly increased in male, female and OVXd female mice 15 days after CAIA-induction (Paper V, Fig 6A, D). On day 54, only male and OVXd female mice had significantly elevated ATF-3 NPs (Fig 6A). There was a marked sex difference in ATF-3 positive DRG NPs at both time points (male > female). Similar to ATF-3, growth associated protein 43 (GAP-43) mRNA levels were only increased on day 15 in male and female CAIA mice (Paper V, Fig 5B). There was a general increase in immunohistochemical staining of GAP-43 in lumbar DRGs in LPS control groups (day 15) and CAIA groups in comparison to the saline control groups (Paper V, Fig 6B, D), which reached statistical significance for CAIA male, female and OVXd mice on days 15 and 54 (Paper V, Fig 6B). No significant sex differences in mRNA or positive NPs for GAP-43 were detected at both time points (Paper V, Fig 5B, 6B).

4.5.5 Expression of galanin in lumbar DRGs and spinal cord dorsal horn

We have conducted qPCR analysis of galanin transcript levels in DRGs in male and female mice after saline injection or CAIA induction. On day 15 after CAIA, the levels of galanin mRNA was significantly elevated in male but not female DRGs (Paper V, Fig 5C). Only few galanin-positive NPs were observed in the lumbar DRGs in the saline treated mice with no differences among the three groups, males, females and OVXd females (Paper V, Fig 6C, D). Induction of CAIA significantly increased the number of galanin-positive NPs in all three groups on day 15 and day 54 (Paper V, Fig 6C, D). There was also a significant sex difference between male and female mice at both time points with males having significantly higher number of galanin-positive NPs (Paper V, Fig 6C). OVX partially reversed such sex difference (Paper V, Fig 6C). Galanin expression was not significantly affected by CAIA induction in comparison to saline group in both male and female spinal dorsal horns (Paper V, Fig 7A, C). A small, but significant, sex difference (male > female) was recorded (Paper V, Fig 7A).

4.5.6 Expression of GFAP and Iba-1 in spinal cord dorsal horn and lumbar DRGs

The intensity of glial fibrillary acidic protein (GFAP) staining in the dorsal horn was significantly increased after CAIA at day 54, but not on day 15 in both male and female mice (Paper V, Fig 7B, C). There was no sex difference in the intensity of GFAP staining after CAIA. Induction of CII Abs and LPS also induced upregulation of the number of cell profiles positive for Iba-1 staining in the lumber DRG on days 15 and 54 after induction of inflammation in both male and female mice (Paper V, Fig 8A, C). There was a significant sex difference on day 15 (female > male) for the number of Iba-1 positive cells in the DRGs, which was however not affected by ovariectomy (Paper V, Fig 8A). Iba-1 positive and CD68 positive cells were colocalized in the DRGs (Paper V, Fig 8D). In CAIA mice there was also an increased intensity of staining for Iba-1 in the dorsal horn in both male and female mice on day 15, but only in female mice on day 54 (Paper V, Fig 8B, C).

5. DISCUSSION

5.1. QUANTITATIVE THERMO TESTING IN RATS WITH SPINAL CORD INJURY

We showed that a Peltier thermode, which is used in quantitative sensory testing in humans, can also be effectively used for quantitative assessment of thermal response threshold in rats with SCI. Thus, we can determine the response threshold temperature for heating and cooling in normal rats and detect the presence of cold allodynia in SCI rats as the temperature required to elicit pain-like response was significantly increased following SCI. The SCI rats did not exhibit increased response to heat stimulation in the same area where mechanical and cold allodynia were observed. These results are similar to clinical findings in patients with SCI, who had hypersensitivity to mechanical and cold, but not to heat stimulation (Finnerup et al., 2003). Lack of heat hyperalgesia has also been reported in several other clinical studies in this patient population (Eide et al., 1996; Defrin et al., 2001).

Recent studies of the TRP family of ion channels have identified two TRP channels, TRPM8 and TRPA1, as the primary targets for sensing cool and noxious cold with activation threshold below 28°C and 17°C, respectively (McKemy et al., 2002 and 2005; Story et al., 2003; Stucky et al., 2009). Since quantitative measurement of cold allodynia in the present study indicated that around 50% of rats showed consistent vocalization threshold above 17°C, which is generally believed to only activate TRPM8 receptors (Foulkes and Wood, 2007; Stucky et al., 2009), it is likely that activation of TRPM8 cold receptors is involved in mediating cold allodynia in spinally injured rats. We have previously reported that treatment of spinally injured rats with a high dose of resiniferatoxin (RTX), which produces a substantial desensitization of capsaicin-sensitive afferents, abolished cold allodynia (Hao et al., 1996). Interestingly, a sub-population of capsaicin sensitive afferents expresses TRPM8 receptors where they coexist with TRPV1 channels (McKemy et al., 2002; Stucky et al., 2009).

A strong positive correlation was found between cold response temperature tested with the Peltier thermode and cold response score tested with ethyl chloride spray. Thus, rats that showed stronger response to a supra-threshold cold stimulation also had higher temperature threshold to cooling (Hao et al., 1996 and 1998; Kouya et al., 2002). A less robust, but significant correlation was found between the response to cold and mechanical threshold. We have shown that mechanical allodynia in SCI rats, was not affected by RTX treatment (Hao et al., 1996). However, we recently found that mechanical allodynia in spinally injured rat is enhanced by topical application of the cold mimetic icilin or menthol (Gao et al., 2013b). Therefore, although the afferents mediating mechanical and cold allodynia may be different in the spinal cord injured rats as judged by their sensitivity to RTX treatment (Hao et al., 1996), it is likely that there are common central mechanisms responsible for the increased responsiveness to mechanical and cold stimulation.

The quantitative method for assessing response temperature increased the reproducibility and sensitivity of the cold test in SCI rats compared to cold scores following stimulation with ethyl chloride. This may be particularly important for future pharmacological experiments. The increased sensitivity of the test can already be seen from current results,

where a sub-population of spinally injured rats that did not develop mechanical allodynia (Xu et al., 1994; Hao et al. 1998; Endo et al. 2008). We have previously reported that these non-allodynic rats did not have cold hypersensitivity as tested with ethyl chloride spray (Hao et al., 1998). However, the present results showed that these rats are hypersensitive to cold compared to normal rats (although less so than allodynic rat), an effect that was not detectable with the cold spray method.

5.2. THE WIDE SPECTRUM ANALGESIC EFFECT OF SINOMENINE

We showed in papers II-IV that a single systemic administration of sinomenine produced antinociception in several rodent models of acute and chronic pain, including against acute heat pain in normal rats, mechanical and heat hypersensitivity in mice subjected to short term inflammation by carrageenan, localized and spread mechanical hypersensitivity in CAIA arthritic mice, mechanical and cold hypersensitivity in mice/rats after sciatic nerve injury and mechanical and cold hypersensitivity in SCI rats. The doses of sinomenine in these studies (up to 80 mg/kg) are in line or lower than those used in previous studies in rodents, mostly for studies on its effects against arthritis (Liu et al. 1996; Huang et al., 2007). Only at the highest i.p. dose of sinomenine, 80 mg/kg, did we observe a mild sedative effect in rats whereas antinociception was observed at 40 mg/kg. No side effects were seen in mice at any dose. Together, the consistent effect of sinomenine across a wide spectrum of models and the lack of interfering side effects suggest that sinomenine is antinociceptive and/or analgesic in rodents.

The analgesic effect of sinomenine has not been well studied before despite some anecdotal clinical evidence for its effects against pain in sciatic neuritis, lumbalgia and muscular rheumatism (Yamasaki, 1976). Wang et al. (2008) showed that in mice 30 mg/kg systemic sinomenine produced moderate antinociception in the tail flick test in mice which agrees with our results in normal rodents. Sinomenine appears to be most effective against pathological pain after inflammation, and particularly, nerve injury. Thus, the profile of analgesia produced by sinomenine is different from that of systemic morphine in our models of nociceptive pain vs. neuropathic pain (Bulka et al., 2002; Yu et al. 1997). The effect of sinomenine may, however, be similar to the effect of dextromethorphan, a non-opioid antitussive that is an NMDA receptor antagonist, which was effective against allodynia after spinal cord injury (Hao and Xu, 1996) while having limited effect in normal rats. It is interesting to note that sinomenine is structurally related to levorphanol and dextromethorphan and while there is currently no evidence that sinomenine can function as an NMDA receptor antagonist, it does have a neuroprotective effect possibly mediated by blocking of acid-sensing ion channel and calcium channels (Wu et al. 2011).

The anti-allodynia effect of sinomenine was not reversed by naloxone at a dose that blocks the three main subtypes of opioid receptors (Handal et al., 1983), suggesting that the effect of sinomenine against neuropathic pain is non-opioid in nature. It has been previous shown that sinomenine can bind to or activate the μ-opioid receptor at high concentration in vitro and the weak anti-nociceptive effect of sinomenine in mice hot plate test can be blocked by naloxone (Wang et al., 2008). We did not examine the effect of naloxone against sinomenine in normal rodents and it is possible that the involvement of opioid receptors is different for the effect of sinomenine between normal and neuropathic states.

5.3. REPEATED SINOMENINE ADMINISTRATION ALLEVIATES CHRONIC NEUROPATHIC PAIN WITHOUT TOLERANCE

We showed that repeated administration of sinomenine produced no signs of tolerance. Furthermore, we observed a significant increase in pre-drug response threshold after two injections, and this effect was maintained for 7 days after the termination of drug administration in SCI rats. In both rats and mice, sinomenine appears to be less effective against cold than mechanical hypersensitivity, which is similar to our previous results (Gao et al., 2013). In addition, no side effects (sedation, motor impairment or irritation) were observed during or after repeated sinomenine administration. Previous studies in rats have also suggested that daily administration of sinomenine at 40 or 80 mg/kg for two weeks did not influence growth, appetite and blood pressure (Zhu, 1998). There were also no apparent withdrawal symptoms following the termination of drug treatment in the present study. These observations, together with the fact that no tolerance to the anti-allodynic effects of sinomenine was observed after repeated administration, suggest that sinomenine may be useful to treat chronic neuropathic pain.

The effects of repeated administration of sinomenine on neuropathic pain-like behaviors in our models are similar to the effect of the anti-epileptics lacosamide and gabapentin (Hao et al., 2000 and 2006; Wu et al., 2004). In particular, the analgesic effect of gabapentin was also increased following repeated administration at doses that were ineffective as a single injection (Hao et al., 2000). Moreover, repeated lacosamide alleviated predrug baseline responses, similar to that of sinomenine (Hao et al., 2006). In contrast, i.p. morphine did not alleviate allodynia in rats with SCI, whereas i.t. morphine did have some anti-allodynic effect, but tolerance was observed after 2 days of twice daily treatment (Yu et al., 1997b). One of the remarkable effects of sinomenine in these two rodent models of neuropathic pain is that it reduced baseline hypersensitivity following repeated administration, resulting in persistent reduction in allodynia. Since sinomenine has a relatively short half-life in rat plasma (Liu et al., 1996; Ling et al., 2005), it is unlikely that this effect is due to an accumulation of the drug following repeated injections. Some of the anti-allodynic effects of sinomenine may be mediated by its metabolites which are known to be present in at least three forms (Cheng et al., 2007). However, it is unknown whether these metabolites are pharmacologically active. Alternatively, the effects of repeated sinomenine administration may reflect sustained physiological changes resulting from repeated drug treatment. Such changes are, however, reversible and may require continuous drug treatment since allodynia recurred within days following the last dose of sinomenine.

The mechanism of action for the anti-allodynic effect of sinomenine in models of neuropathic pain is not clear. As mentioned above the anti-allodynic effect of sinomenine was not reversed by the opioid receptor antagonist naloxone (paper II) and the profile of analgesia produced by sinomenine is different from that of systemic morphine (Bulka et al., 2002; Yu et al., 1997 and 1997b). In contrast, the effect profile of sinomenine is similar to that of dextromethorphan, a non-opioid antitussive which is a weak noncompetitive NMDA receptor antagonist (Hao and Xu, 1996). In accordance, we found repeated administration of sinomenine delayed tolerance to morphine (Wang et al., 2002; Wang et al., 2003), which was also observed with dextromethorphan (Elliott, 1994). One of the possible mechanisms for the anti-allodynic effect of chronic sinomenine may be related to its ability to modulate neurotransmitter release in the spinal cord and brain. Systemic sinomenine alters the level of monoamines in extracellular fluid in the striatum in rats after sciatic nerve injury with increase in the level of noradrenaline and decrease in

level of dopamine and serotonin (Zhang et al., 2013). These effects are correlated with analgesic effect of sinomenine (Zhang et al., 2013). Chronic SIN may produce long term effects on transmitter synthesis and neuronal functions through altered transmitter release. Sinomenine also has distinct immunoregulatory and neuroprotective properties. It can reduce the production of cyclooxygenase (COX)-2 dependent prostaglandin E2 (PGE2) (Liu et al., 1994), as well as block nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and p38 mitogen-activated protein kinases (p38MAPK) signal pathways (Wang et al., 2005; Huang et al., 2008). It is conceivable that some of these properties may reduce neuronal sensitization contribute to sinomenine's analgesic effects in neuropathic pain.

5.4. THE EFFECT OF SINOMENINE ON EXPERIMENTAL RHEUMATOID ARTHRITIS

Sinomenine is used in China and Japan as an anti-rheumatic drug (Yamasaki, 1976). The efficacy of sinonemine against RA is well established in rodent RA models (Liu et al. 1996; Huang et al. 2007). Clinically, sinomenine was more effective than NSAIDs in ameliorating morning stiffness, painful joints and erythrocyte sedimentation rate in RA patients (Xu et al., 2008). The mechanism of the effect for sinomenine against RA has been suggested to be due to its ability to reduce the production of proinflammatory cytokines by suppressing the activation of NF-κB (Wang et al., 2005; Zhou et al., 2008; Cheng et al., 2009) and to inhibit key inflammatory mediators such as TNF and IL1-β (Wang et al., 2005).

In the present study, we did not find that repeated sinomenine administration (2 /day for 5 days) reduced acute inflammation in the CAIA mice. This could be due to several factors, such as dose, timing of the treatment and models used. In contrast to the CIA model, which requires T-cell activation, the CAIA model, by directly injecting antibodies against the type II collagen to trigger arthritis, bypasses this step. Thus, the anti-rheumatic effect of sinomenine may be related to inhibition of T-cell activation. Administration of sinomenine effectively and dose-dependently alleviated the localized and spread mechanical hypersensitivity during both phases without producing side effects. Furthermore, repeated administration of sinomenine during the peak of inflammation did not change the arthritic scores, despite producing marked analgesia. Thus, it is likely that the analgesic mechanism of sinomenine is independent from possible anti-inflammatory action of the compound.

No tolerance was seen to the analgesic effect of sinomenine following repeated administration. Lack of tolerance to the effect of sinomenine was similarly noted in rodent models of neuropathic pain (Gao et al., 2014). It has also been shown that long-term pretreatment with sinomenine may delay the analgesic tolerance to morphine (Wang et al., 2008). Sinomenine is not an opioid and the anti-nociceptive effect of sinomenine in neuropathic pain is not mediated by naloxone sensitive opioid receptors (Gao et al., 2013). However, sinomenine can interact with neuro-immune crosstalk by suppressing microglia activation (Qian et al., 2007; Shukla and Sharma, 2011). Since microglial activation was found in the spinal cord of CAIA but not control mice (Bas et al., 2012), it is possible that downregulation of microglial activities in the spinal cord by sinomenine is responsible for the reduction pain-related behavior in the CAIA model.

Sinomenine also produced a range of other pharmacological effects in the central and peripheral nervous system and these effects may also be involved in the analgesic

mechanisms of this compound. These effects include immunoregulative properties and actions on systems such as histamine, proinflammatory cytokines, COX2 dependent PGE2, interferon gamma (INF-γ), reactive oxygen species (ROS), nitric oxide (NO), NF-κB, p38MAPK, metalloproteinases (MMPs) and TNF. Further identification of the effects of sinomenine on neuronal and immune systems may shed lights in understanding of the mechanisms of analgesia by sinomenine.

5.5. SEX DIFFERENCES IN THE DEVELOPMENT OF ARTHRITIS AND PAIN BEHAVIORS IN CAIA MICE

Similar to previous findings (Nandakumar and Holmdahl, 2007; Bas et al., 2012), we showed that in the CBA strain of male and female mice, CII antibodies with LPS immunization triggered inflammatory responses in the fore- and hind paws. The extent of inflammation, as measured by the arthritis scores, is more profound in the forepaw than in the hind paw, which has also been observed for the CBA strain of mice previously (Bas et al., 2012). CBA mice of both sexes also developed marked mechanical hypersensitivity in the fore- and hindpaws following a similar time course as the development of inflammation. However, the duration of mechanical hypersensitivity for both sexes was markedly longer than that of inflammation. Thus, while the peak of inflammation was reached at around day 15 and inflammation subsided after day 30, the mechanical hypersensitivity persists up to day 54 which is the cut-off time of observation. Similar disparity between inflammation and hypersensitivity has also been observed previously in this model in several strains of mice (Bas et al., 2012) and in other models of RA (Christianson et al., 2011). In addition to mechanical hypersensitivity detected in fore- and hindpaws, we have also observed persistent mechanical hypersensitivity in mice with CAIA at the neck and upper back regions. This may be similar to the neck and back pain observed in RA patients resulting from arthritis in the spine (Rawlins et al., 1998). Moreover, this may also be related to generalized pain conditions, such as nonarticular rheumatism (Moreland and Curtis, 2009). Further, it has been reported that in RA patients with long disease duration (>5 years) there was an increased sensitivity to pressure both in structures overlying the inflamed joint and in non-inflamed tissues (Leffler et al., 2002).

One of the significant differences between human RA and the widely used rodent model of collagen-induced arthritis is the direction of sex differences with rodent studies showing that the development of arthritis is more prevalent in males (Holmdahl et al., 1989; Jansson et al., 1994). It has been shown in a recent study that in a strain of transgenic mice that lacked all endogenous mouse class II genes and expressed the RA susceptibility allele HLA-DRB1*0401, collagen induced arthritis developed predominantly in females (Taneja et al., 2007). We observed similar sex differences in inbred CBA strain of mice after CAIA with females exhibiting significantly higher arthritis scores as well as more severe localized and spread hypersensitivity to mechanical stimulation. These results support the notion that CAIA is a clinically relevant mouse model to study sex differences in RA and arthritic pain behaviors. The sex difference in arthritis score and mechanical hypersensitivity is reduced in OVXd female mice, suggesting a possible role for female sex hormones in such sex differences. The relationship between sex hormones and pain has been extensively studied and many chronic pain conditions have been shown to be influenced by the level of female sex hormones, including changes following the menstrual cycle (LeResche et al., 2003; Brandes, 2006; Martin and Lipton, 2008) as well as at the menopause (Pamuk and Cakir, 2005; Greenspan et al., 2007; Cairns and Gazerani, 2009). On the other hand, the relationship between female sex hormones and RA appears to be complicated (Jansson and Holmdahl, 1998; Whitacre, 2001; Cutolo et al., 2002; Islander et al., 2011).

Ovariectomy was performed in mice 20 days after birth in the current study. Our results thus suggest that depleting estrogen circulation at an early age can alter the disease progression profile in CAIA. Regardless of the possibly diminished bone protection by losing estrogen signaling (Imai, 2013; Ohlsson and Vandenput, 2009), OVXd females still had less severe CAIA symptoms than females, suggesting that other key events such as epigenetic and developmental modifications following ovariectomy may contradict estrogen signaling and modulate sex differences in CAIA. In agreement with the present results, we have shown previously that blockade of estrogen signaling by genomic knockout of estrogen α or β receptor induced alterations in sensory functions that making female mice more resistant to inflammatory pain (Li et al., 2009).

5.6. SEX RELATED PHENOTYPIC CHANGES IN DORSAL ROOT GANGLIA AND SPINAL CORD IN CAIA MICE

No changes in expression of SP and CGRP in lumbar DRG and spinal cord dorsal horn were observed in the inflammatory or post-inflammatory phase in mice after CAIA, nor did we observed sex difference in the expression of SP and CGRP . These results suggest that these two peptides are not prime mediators of CAIA induced hypersensitivity and its sex differences.

In contrast, the neuropeptide galanin was significantly upregulated in the DRG seen both at the mRNA level as well as at the protein level studied by immunohistochemistry. Interestingly, galanin level also showed significant sex difference with higher level of expression in male mice and such sex difference is partially reversed by ovariectomy. The role of galanin in pain modulation at the spinal cord level has been extensively studied, and it is now generally agreed that spinally applied galanin produces a biphasic dose-dependent effect on pain sensitivity through activation of inhibitory galanin receptor 1 or excitatory galanin receptor 2 respectively (Xu et al., 2008, 2010). Endogenous galanin has primarily an inhibitory (Xu et al., 2008), particularly after peripheral nerve injury when the synthesis of galanin is increased in sensory neurons and such increase is correlated to the suppression of mechanical hypersensitivity (Hökfelt et al., 1994; Shi et al., 1999). This notion has been confirmed in transgenic mice that overexpressing galanin in the DRG after sciatic nerve axotomy. Phenotypic analysis revealed markedly attenuated allodynia when galanin was overexpressed and an increase in allodynia following galanin suppression (Pope et al., 2010). Our current results suggest that galanin may also be involved in the modulation of pain sensitivity in CAIA, where an increased level of galanin expression in DRG, in response to the systemic inflammation, may help to counteract the increase in pain response. Furthermore, the level of galanin was significantly higher in male mice than in females after CAIA, correlating with reduced pain response in male mice. Interestingly, galanin upon release in the periphery including joints may also have anti-inflammatory functions (Lang and Kofler, 2011). Hence, sex-dependent differential regulation of galanin in the DRG may partially explain sex difference in the development of inflammation and pain in the CAIA model in CBA mice and it may be suggested that drugs that activate galanin receptor 1 are analgesic in pain associated with RA in female patients. Galanin may also be involved in neuronal immune interaction counterregulating acute phase inflammation and suppressing excessive inflammatory cytokines (TNF-α and IL-1β) through galanin receptor 2 (Lang and Kofler, 2011) in addition to its direct effect on pain suppression via galanin receptor 1 (Hao et al., 1999). To what extent such effect of galanin is involved in the sex differences in joint inflammation in the CAIA model remains to be determined. The underlying mechanisms of the sex-dependent differential regulation of galanin in sensory neurons in CAIA mice are unclear. Early studies have demonstrated that the galanin gene in some tissues is

sensitive to physiological levels of estrogen (Vrontakis et al., 1987; Kaplan et al., 1988). However, no sex difference in the level of galanin is seen in normal DRG. Whether or not conditions such as RA could change the sensitivity of tissues towards estrogen remains to be determined.

Spinal glial activation was seen in both phases of CAIA (Bas et al., 2012), indicating that sensitized spinal glial cells may lead to the enhancement of excitatory synaptic transmission (Ji et al., 2013). In addition, similar to early findings with CAIA and K/BxN serum transfer arthritis models (Christianson et al., 2011; Bas et al., 2012), astrocyte activation in the dorsal horn of spinal cord was seen in the post-inflammatory phase of CAIA as a secondary event to microglia activation, which may contribute to the maintenance of mechanical pain (Spataro et al., 2004). In DRG, Iba-1 positive cells share the properties of macrophages since they are usually colocalized with CD68. It has been previously suggested that activated macrophages may be involved in peripheral sensitization in inflammatory and neuropathic pain models (Dubový et al., 2007; Ji et al., 2013). In the present study, we found that Iba-1 expression was up-regulated in the DRG in mice following CAIA. Furthermore, the number of Iba-1 positive cells was significantly higher in female than in male DRGs. This would indicate that macrophage activation in the DRG may also be involved in hypersensitivity and its sex differences following CAIA. Macrophages infiltration and activation can be triggered by local inflammatory events via toll-like receptor 4, NFκB, TNF or IL-1β signaling (Ji et al., 2013). It is also interesting to note that galanin, as well as ATF-3 can act as the anti-inflammatory regulators (Gilchrist et al., 2006; Thompson et al., 2009; Pope et al., 2010; Lang and Kofler, 2011). Hence, the sex dependent changes in the DRG expression of galanin, ATF-3 and Iba-1 after CAIA may be related to each other and play an important role in CAIA induced pain.

In my hypothesis illustrated in Fig. 3, I propose that genomic, hormonal and developmental factors together influenced the development of sex difference in CAIA CBA mice, with females having more pronounced acute inflammation and chronic mechanical allodynia. CAIA induces upregulation of galanin and ATF-3 in DRG neurons and Iba-1 in macrophage-liked cells. These markers are affected by estrogen signaling (male > female for ATF-3 and galanin, male < female for Iba-1). In peripheral tissue, ATF-3 is generally believed to induce anti-inflammatory effects. Its mechanism is unclear, however, and may be involved in the modulation of neuronal releases of ATP and cytokines which may further inhibit toll-like receptor signaling and NFκB activation in macrophage-like cells surrounding these neurons. It is known that increased local expression of galanin can induce extracellular levels of TNF and IL-1β in DRG (via galanin receptor 2), and at the spinal cord level, galanin has an analgesic efficacy mediated by GalR1. Taking together, induction of ATF-3 and galanin in DRG following CAIA may have suppressive effect on macrophage activation (stained by Iba-1), and result in lower peripheral sensitization in males than females.

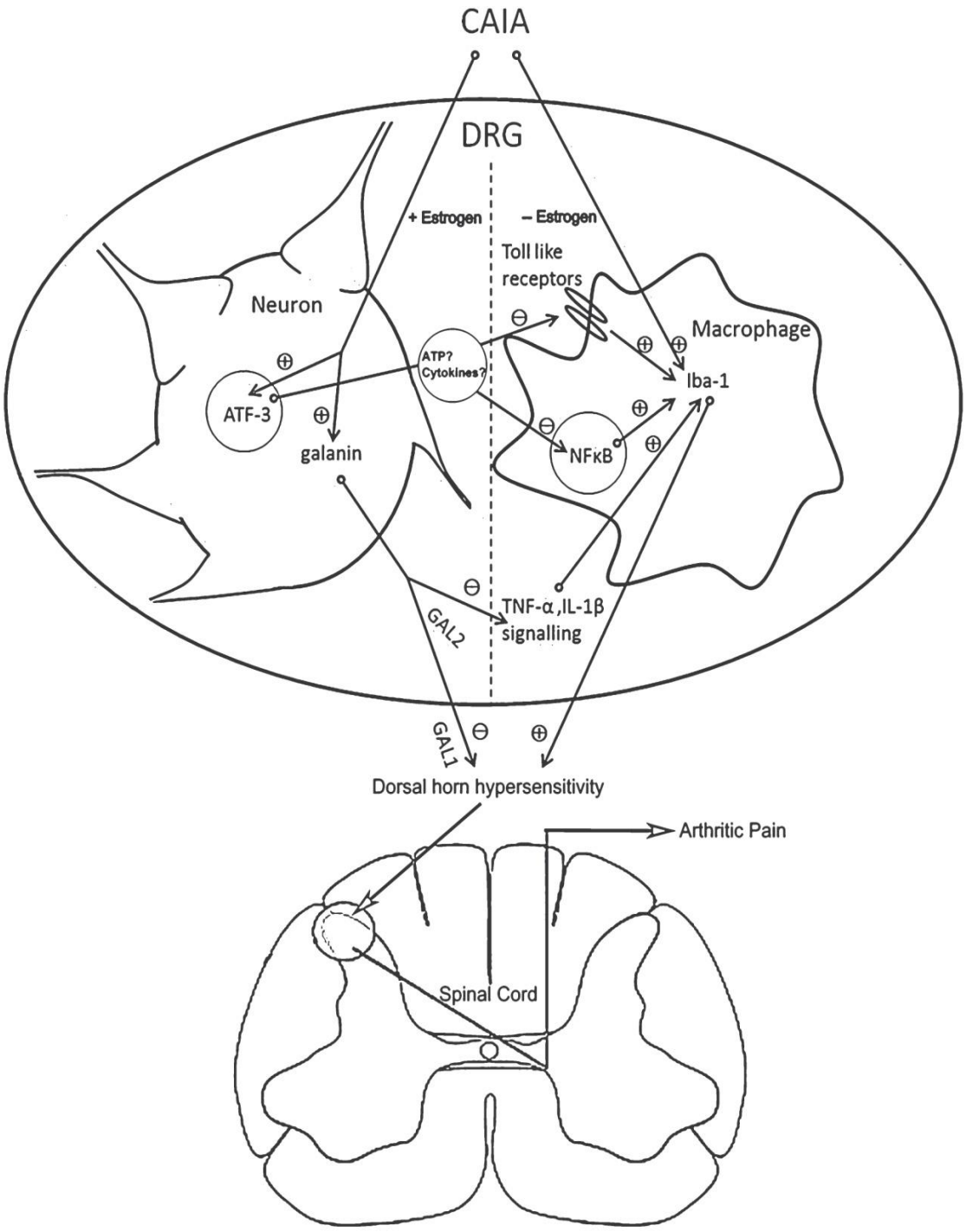

Fig. 3 A schematic illustration of hypothesis of sex related neuro-immune interaction in DRG and spinal cord in CBA mice with CAIA.

6. CONCLUSIONS

1. Quantitative testing of responsiveness to cold using a Peltier thermode increases the detecting cold allodynia and could be used as a valuable tool in studies of the mechanisms and treatment of cold allodynia in neuropathic pain.

2. Sinomenine produced anti-nociceptive and analgesic effects in a broad spectrum of acute and chronic pain models in rats and mice at doses that did not produce observable side effects.

3. The anti-allodynic effect of sinomenine upon repeated chronic administration did not lead to tolerance, but rather enhanced its effect, in two rodent models of neuropathic pain and in a mouse model of arthritis. This leads to a persistent, but reversible, analgesia with no observable side effects. The results from this thesis may suggest potential clinical application of sinomenine as a novel analgesic in treating chronic neuropathic and arthritic pain.

4. After the induction of CAIA there is a significant sex difference in the level of joint inflammation and in associated mechanical hypersensitivity in the CBA mouse strain. This is similar to the clinical situation with females having more profound arthritis and pain. Further, we showed that such sex differences are correlated with the alteration of neuronal expressions of galanin and ATF-3, and the macrophage marker Iba-1 in DRG.

5. Our findings suggest that the CAIA model in certain mice strain may be a clinically relevant model for studying the mechanisms of sex difference in arthritis and arthritis-induced pain which may involve neuro-immune crosstalk.

7. ACKNOWLEDGEMENTS

I would like to thank the Department of Physiology and Pharmacology, Karolinska Institutet and express my gratitude to all the people who helped me in my life as a doctoral student. In particular, I would like to thank:

My supervisor, Docent Xiao-Jun Xu. Thank you for accepting me as a student, introducing me to the field of pain research, for your generosity in helping me in every aspect of work and life, for your enthusiastic discussion and advices about science and carrier. Your constructive ideas led me in my development as a better scientist. Working with you has been a great honor.

My co-supervisor, Prof. Zsuzsanna Wiesenfeld-Hallin. Thank you for giving me this opportunity to work in your lab. Your outstanding leadership and excellent scientific ideas, as well as your great encouragement and perfect pace-control of the doctoral study process are invaluable supports for me. Thank you for the family-like working environment that you have created. Also the wonderful times we had together outside work are definitely unforgettable! Special thanks to Associate Prof. Rolf Hallin, for your hospitality and kindness during every visit to your home and summer house.

My co-supervisor, Lektor Camilla I Svensson, thank you for giving me the chance to participate in your research activities, and the possibility to continue my unfinished project in your lab. Your constructive discussions not only increased my knowledge but also inspired me and had profound impact on me in science. You have created a great research atmosphere and encouraged me with warm support. Working with you has been a great journey. Thank you, Dr. Simone Codeluppi, I appreciated a lot for your kindness and humor. The time spent with you is extremely fun.

Dr. Jing-Xia Hao, my tutor in experimental techniques. Thank you for passing and sharing your excellent skills, and your advices in helping to solve practical matters. Also, thank you for taking care of me and Yang. I would never forget your spectacular performances of Peking Opera, the fantastic traditional Chinese art.

Prof. Stefan Eriksson, head of the Department of Physiology and Pharmacology, thank you for taking care of me and creating a great working environment in the Department.

Prof. Kaj Fried, thank you for being my study external mentor. It is always exciting whenever we had talks during lunches.

Prof. Tomas Hökfelt, thank you for your constructive advices in SSF project, and your consistent help in refining the manuscript, it is a great honor to know you and be able to work with you.

Dr. Tiejun Shi, thank you for teaching different experimental and microscopic techniques. Every time after we talk, I feel that I have learned a lot and being refreshed.

Dr. Malin Lagerström, Department of Neuroscience, Uppsala University, thank you for the introducing me the great project which opens a door for me to further develop myself in neuroscience. And thank you for giving me the potential opportunity to be a post-doc in your lab. It is a strong inspiration for me to build my scientific career.

Prof. Sharon Elander, thank you for your help in the study designing and deciding the schedule for microPET project, and especially for the discussion and encouragement about my thesis defense. Dr. Lu Li, thank you for your state of art microPET technical assistance, and your beautiful and delicious homemade moon cakes.

Prof. Fred Nyberg, it is a great pleasure to cooperate with you, thank you for your exciting discussions with us, I learned a lot from you. Anna Carlsson-Jonsson, it is a fantastic experience to have successful collaboration with you. I was inspired by your dedication in science, and thank you for visiting me in your leisure time during vacation.

Prof. Ernst Brodin, thank you for being my half time committee member, and I really enjoyed a lot in the PAIN CLUB organized by you. Lektor Inger Johansson, thank you so much for your assistances in my doctoral education related procedures and teaching activities.

Past members of the group: Dr. Lili Li and Jinling Huang, thank you for being such a nice friend like big sister and brother for me. Dr. Cecilia Dominguez and Dr. Anna-Karin Persson, thank you for your kindnesses to me when I first arrived Sweden, and thank you for teaching me some basic Swedish.

Tiansheng Shi, thank you for your friendship both at and after work, and your generous helps. I wish you all the best, and I am sure a great future is waiting for you.

I would like to thank all the members in CIS group, and the big SSF group. In particular Azar Baharpoor, Duygu Bas, Katalin Sandor, Su Jie, Gustaf Wigerblad, Ada Delaney, Sally Abdelmoaty, Nilesh Agalave, Jungo Kato, Kim Kultima, Camilla Ultenius, Alex Bersellini Farinotti, Teresa Fernandez Zafra, associate Prof. Jon Lampa, Lektor Eva Kosek, Erwan Le Maitre, Sofia Martinsen, Swapnali Barde and Reem Altawil. Thank you for your generous help in experiments and exciting discussions, collaborating with you is very honorable.

I would like to thank all the administration and faculty personnel at department of Physiology and Pharmacology, in particular, Camilla Fors Holmberg, Freddie Hellström, Renée Andersson, Peter Wolf, Micke Elm, Sofia Pettersson, René Rizzo, Ylva Haraldsdotter, Sarah Lindholm, Kent Jardemark and Prof. Håkan Westerblad. Thanks that you are always ready to help with when there are problems.

All the colleagues in the Department of Physiology and Pharmacology, especially, Dr. Gunnar Schulte, Dr. Andrei Chagin, Dr. Eva Lindgren, Julian Petersen, Jacomijn Dijksterhuis, Jia Guo, Xicong Liu, Miyoung Lee, Karuna Vuppalapati, Shane Wright, Marketa Kauka, Elisa Arthofer, Michaela Kilander, Xiaojing Tang, Thibault Bouderlique, Phillip Newton, Lei Li and Ming Liu, thank you for your encouragement.

I would like to thank Prof. Jinping Li, associate Prof. Xiao Zhang, Dr. Jiang Yu, Dr. Hongmei Yan, Dr. Gang Liu, Dr. Xun Wang, Huijie Li, Jingjiao Wang, Sichao Li, Xiao Zhan, Yun Zhang, Zhiyang Song, Shouting Zhang and many other CBAS members. Thank you for your encouragement, friendship, and happy memories we had together.

Special thanks to my parents: thanks for all your love, wisdom and support!

Qiongyuan, my wife, thank you for all your love and support! Meeting you has been most wonderful, I love you!

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