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Sex differences in acute and chronic experimental pain models

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To my family and the memory of my grandfather

ABSTRACT

Sex differences in response to noxious stimuli and in the development of chronic pain have been increasingly recognized and studied in humans and laboratory animals. In the clinic, extensive evidence indicates that there are a large number of chronic pain conditions that have higher prevalence in women than in men and some of these conditions, such as migraine and temporomandibular pain disorder, are also affected by the menstrual cycle. The mechanisms for sex differences in pain, particular in chronic pain, are largely unknown. The work presented in this thesis aims to explore mechanisms of sex differences using experimental pain models in rodents.

In the first part of the thesis, we explored the role of estrogen receptors and the adenosine A_{2A} receptor in sex differences in pain using genetically modified mice. We observed that in wild-type controls for estrogen receptor α or β knock-outs, females were significantly more sensitive than males to mechanical stimulation under normal condition and after carrageenan-induced inflammation. Such sex differences were eliminated in mice lacking either estrogen receptor α or β. Mechanical hypersensitivity resulting from partial sciatic nerve injury did not however differ between the sexes or between the wild-types and both estrogen receptor knock-outs. These results suggest that estrogen receptors α or β play a role in sex difference in basal mechanical pain threshold and inflammatory hypersensitivity. There was no sex difference in baseline mechanosensitivity in mice lacking the adenosine A_{2A} receptor and wild-type controls. Carrageenan-induced mechanical hypersensitivity was significantly reduced in the A_{2A} receptor knock-outs compared to wild-types. ZM-241,385, a selective A_{2A} receptor antagonist, reduced inflammatory hypersensitivity in wild-type females, but not in males. The selective A_{2A} receptor agonist CGS 21680 produced significantly more hypersensitivity in wild-type female mice than in males. These results suggest that activation of peripheral adenosine A_{2A} receptors contributes to inflammatory hypersensitivity and that this effect is more prominent in females than in males.

The second part of the thesis deals with the development of neuropathic pain-like behaviors (allodynia) in rats after spinal cord or infraorbital nerve injury. We observed a significant sex difference (females > males) in the development of mechanical allodynia after spinal cord injury in rats independent of the extent of injury. Increased mechanical hypersensitivity in females was not related to estrous stage at the time of injury. Similarly, after infraorbital nerve injury, female rats developed more persistent local and spread mechanical allodynia which was also not influenced by the estrous stages at the time or after injury.

These studies provide further evidence for the presence of sex difference in baseline mechanical pain threshold, inflammatory hypersensitivity and experimental neuropathic pain in rodents. Furthermore, our results showed that estrogen receptors and adenosine A_{2A} receptor may be involved in sex difference in pain sensitivity under normal condition or after inflammation. Finally, although female rats developed more persistent allodynia-like behaviors after spinal cord or infraorbital nerve injury, there appears to be no impact of the estrous cycle on pain development.

Key words: estrogen, adenosine, sex difference, nociception, carrageenan, inflammation, neuropathic pain, mice, rats

LIST OF PUBLICATIONS

I. **Li L**, Fan X, Warner M, Xu XJ, Gustafsson JÅ, Wiesenfeld-Hallin Z. Ablation of estrogen receptor α or β eliminates sex differences in mechanical pain threshold in normal and inflamed mice. Pain. 2009;143:37-40.

II. **Li L**, Hao JX, Fredholm BB, Schulte G, Wiesenfeld-Hallin Z, Xu XJ. Peripheral adenosine A_{2A} receptors are involved in carrageenan-induced mechanical hyperalgesia in mice. Neuroscience. 2010;170:923-928.

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IV. **Li L**, Gao T, Hao JX, Xu XJ, Wiesenfeld-Hallin Z. The increased mechanical allodynia-like response after infraorbital nerve injury in female rats is not dependent on estrous stage. Manuscript.

Contents

LIST OF ABBREVIATIONS

A_1R	Adenosine A_1 receptor
$A_{2A}R^{-/-}$	Adenosine A_{2A} receptor knock-out
$A_{2A}R^{+/-}$	Adenosine A_{2A} receptor heterozygous
$A_{2B}R$	Adenosine A_{2B} receptor
A_3R	Adenosine A_3 receptor
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
AP1	Activator protein 1
ASIC	Analgesics–acid-sensing ion channels
cAMP	Cyclic adenosine monophosphate
CP	Caudate putamen
CNS	Central nervous system
COX	Cyclooxygenase
DRG	Dorsal root ganglion
$ER\alpha^{-/-}$	Estrogen alpha receptor knock-out
$ER\beta^{-/-}$	Estrogen beta receptor knock-out
ERE	Estrogen response element
ES	Embryonic stem
IASP	International Association for the Study of Pain
IBS	Irritable bowel syndrome
IL-6	Interleukin-6
i.p.	Intraperitoneally
i.v.	Intravenously
LH	Luteinizing hormone
MAD	Median absolute deviation
Mclr	Melanocortin-1 receptor gene
NASIDs	Non-steroid anti-inflammatory drugs
NGF	Nerve growth factor
NRM	Nucleus raphe magnus
PAG	Periaqueductal gray
PBS	Phosphate buffer saline
RA	Rheumatoid arthritis
s.c.	Subcutaneously
SEM	Standard error of the mean
TMD	Temporomandibular disorder
WDR	Wide dynamic range

1 INTRODUCTION

1.1 PAIN: DEFINITION AND CLASSIFICATION

Pain normally serves as an alarm system activated in response to impending damage to the organism. According to 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). It is a multidimensional experience, which contains essentially a sensory, cognitive and emotional component (Woolf, 2004).

Pain can be classified into different categories according to various criteria, such as those based on the cause of pain, its duration, location, underlying diseases etc. The most widely used criteria are aetiological (i.e. based on the cause of pain). In this regard, pain can be classified into three major categories, nociceptive, inflammatory and neuropathic. Nociceptive pain is generated by activation of nociceptors that are specialized to be activated by noxious stimuli which have the potential of causing tissue damage. Nociception or nociceptive pain is essential in survival for organisms to avoid potential or actual tissue damage (Scholz and Woolf, 2002). Nociceptive pain is mostly recognized as acute pain since that pain stops when the stimulus has been removed.

Inflammatory pain is associated with processes that can be caused by tissue damage, infections, tumor growth and various forms of chronic inflammatory diseases, such as autoimmune disease. During inflammation, multiple mediators are released locally from the damaged and recruited inflammatory cells. This results in the release of cytokines, growth factors, neuropeptides, kinins, purines, amines, prostanoids and ions, including protons (Boddeke, 2001; Manthy et al, 2002). These mediators can activate and sensitize nociceptors, thus evoke pain (Scholz and Woolf, 2002). The symptoms of inflammation include cell migration, oedema, erythema, pain and hyperalgesia (Marchand et al, 2005). In most of the cases, inflammatory pain responds to non-steroid anti-inflammatory drugs (NASIDs) or opiates such as morphine (Basbaum et al, 2008; Fitzcharles et al, 2010). Inflammatory pain under many conditions such as rheumatoid arthritis (RA) is chronic. Chronic inflammatory pain can be characterized by hyperalgesia (greater pain after normally painful stimuli) and allodynia (normally non-noxious stimuli that are perceived as painful).

Neuropathic pain arises from a primary lesion or dysfunction in the peripheral or central nervous system (CNS) (Merskey and Bogduk, 1994). Examples of neuropathic pain include painful polyneuropathy, postherpetic neuralgia, trigeminal neuralgia, spinal cord injury pain and post-stroke pain. Clinically, neuropathic pain is characterized by spontaneous ongoing or shooting pain and evoked pain such as hyperalgesia and allodynia (Baron et al, 2010). Neuropathic pain is mostly chronic, difficult to treat and associated with plasticity in the central and peripheral nervous system. The mechanisms of neuropathic pain are not well understood and treatments are largely unsatisfactory. Neuropathic pain may respond to some antiepileptics, tricyclic antidepressants, and antiarrhythmics. Local anesthetics used to block the nerve may also be effective in some cases.

1.2 PAIN PATHWAYS

Nociception is initiated when noxious stimuli, which may be mechanical, thermal or chemical, are detected by nociceptors (Basbaum and Jessell, 2000). The cell bodies of the nociceptors are located in the dorsal root ganglion (DRG) and the trigeminal ganglions and they have peripheral and central axons that innervate their target organ and the spinal cord, respectively (Basbaum et al, 2009). There are two major categories of nociceptors, Aδ fibers and C fibers (Meyer et al, 2008). Aδ fibers are thinly myelinated and respond to mechanical and thermal stimulation. These fibers differ considerably from larger diameter Aβ fibers which respond to innocuous mechanical stimulation (i.e., light touch). C fibers are unmyelinated, and many of them are polymodal which means that they respond to stimulation of different modalities such as heat, mechanical stimuli, protons and cold (D'Mello and Dickenson, 2008). The energy of noxious stimuli is converted into electrical activity by these receptors and conducted to the spinal cord where the central axons of sensory nerves terminate (Scholz and Woolf, 2002).

The dorsal horn of the spinal cord is the receiving zone for primary afferent axons that transmit signals from nociceptors. In general, Aδ fibers and C fibers terminate in the superficial layers, laminae I and II, although some reach deeper laminae (Light and Perl, 1979; Sugiura et al, 1987). Large myelinated fibers including Aα and Aβ fibers project to the deeper laminae (laminae III-VI) (Brown, 1981). A large number of neurons located in the dorsal horn are capable of responding to input from primary afferents. Among these are nociceptive specific neurons located primarily in lamina I of dorsal horn which

respond exclusive to activation of nociceptors, and wide dynamic range (WDR) neurons (Kandel and Schwartz, 1991), located mostly in the deeper laminae of the dorsal horn. WDR neurons respond to stimulation of various sensory modalities (thermal, chemical and mechanical) and in a graded fashion in accordance to the intensity of stimulation (Cervero et al, 1976).

From the spinal cord, the nociceptive information is conveyed to the thalamus via the contralateral spinothalamic tract, to the medulla and brainstem via the spinorecticular and spinomesencephalic tracts or to the hypothalamic nuclei via the spinohypothalamic tract. Ascending information is further transmitted to several cortical and subcortical brain regions including anterior cingulate cortex, insular cortex, frontal and pre-frontal cortices, primary and secondary somatosensory cortices, and amygdala (Tracey, 2005). Brain structures such as periaqueductal gray (PAG) and the nucleus raphe magnus (NRM) also modulate pain transduction by either inhibiting or facilitating spinal nociceptive input (Porreca et al, 2002).

1.3 SEX DIFFERENCES IN PAIN AND THE ROLE OF SEX HORMONES

As a complex and multidimensional sensory experience, pain responses of different individuals can be affected by a broad range of variables, such as genetic, physiological, psychological, cultural and social factors (Holdcroft and Berkley, 2005; Greenspan et al, 2007). In recent years, sex has been shown to be an important factor in modulating the experience of pain with a growing body of evidence demonstrating that males and females experience pain differently and respond differentially to specific classes of analgesics (Paller et al, 2009). In both experimental and clinical studies, pain thresholds and pain tolerance are generally shown to be lower in females than males as tested with various stimulus modalities (Berkley, 1997; Fillingim et al, 1999; Mogil, 2000; Barrett et al, 2002; Wiesenfeld-Hallin, 2005; Greenspan et al, 2007). Females report also more frequent, severe and longer lasting pain which is often anatomically more diffused than males with similar disease processes (Berkley, 1997; Hurley and Adams, 2008). There are many chronic pain states such as migraine, temporomandibular disorder (TMD), rheumatoid arthritis (RA), irritable bowel syndrome (IBS), and fibromyalgia that are more prevalent in women (Ektor-Andersen et al, 1993; Craft et al, 2004; Holdcroft and Berkley, 2005; Greenspan et al, 2007). However, some chronic conditions such as migraine without aura and cluster headache are more common in men (Greenspan et al, 2007).

Imaging studies of the brain have shown sex differences in humans in the intensity of cerebral activation and the spatial pattern in response to acute noxious stimuli (Paulson et al, 1998; Derbyshire et al, 2002; Zubieta et al, 2002; Moulton et al, 2006).

The response to analgesics has also been shown to be sex dependent (Craft, 2003; Wiesenfeld-Hallin, 2005; Berkley et al, 2006). In humans, women have greater response to μ-opioid agonists than men (Kest et al, 2000; Cook et al, 2000; Craft, 2003) whereas in rodents, morphine appears to be more effective in males than in females (Mogil et al, 2000; Berkley et al, 2006). The response to κ-opioids is also different between sexes in both mice and human (Wiesenfeld-Hallin, 2005). κ-Receptor agonists appear to be more potent analgesics in women than in men (Gear et al, 1996 and 1999) which may be due to pain modulation by the melanocortin-1 receptor (Mclr) gene in a female-specific manner in both mice and human (Mogil et al, 2003). Besides opioids, responses to other analgesics also show sex differences. For example, administration of an inflammatory agent to cyclooxygenase-1(COX-1) and cyclooxygenase-2 (COX-2) knock-out mice had greater effects in females (Chillingworth et al, 2006). Similarly, Chanda and Mogil (2006) found that a non-specific analgesics-acid-sensing ion channel (ASIC) blocker (amiloride) had greater analgesic effect in female mice on formalin-induced nociception.

Sex hormones (androgens, estrogens and progestagens) have received considerable attention in research of the mechanisms of sex differences in pain. The levels of plasma estrogen vary throughout the menstrual cycle during the reproductive years (18-50 years) (Fig.1a) and they are on average 3–10 times greater than those found in men (Eldrup et al, 1987). It has been shown that in many chronic pain conditions, there is a correlation between pain and the menstrual cycle. For example, about 10% of female migraine sufferers have menstrual migraines, whereas headaches regularly occur at the beginning of menstruation (Brandes, 2006; Martin and Lipton, 2008). The highest severity of TMD pain was noted during the late luteal and early follicular phases. There was also a second peak of increased pain severity that occurred around ovulation in normally cycling women (LeResche et al, 2003). Menopause, which results in a decrease in estrogen levels in women, is strongly associated with changes in musculoskeletal pain conditions. The number of women with RA and fibromyalgia increases around menopause, whereas the number of women suffering from migraine and TMD decreases (Pamuk and Cakir, 2005; Greenspan et al, 2007; Cairns and Gazerani, 2009). A number of studies have also examined changes in pain in response to noxious stimuli across the menstrual cycle in

human. A meta-analysis of these studies indicates that for most forms of painful stimuli (with exception of electrical stimuli), higher pain thresholds and tolerance are observed during late follicular and luteal phases, and the effect size is small to moderate (Riley et al, 1999).

The female rat has a 4-5 day vaginal estrous cycle which is correlated with changes of hormone levels (Fig.1b). The estrous cycle in rats is generally divided into 3 stages: proestrous, estrous and diestrous (Krinke, 2000). In cycling females, serum estradiol concentration is low during estrous, gradually rises during diestrous, peaks during early proestrous, and declines during late proestrous. Serum progesterone concentration peaks twice, once during middle diestrous and a second time during late proestrous (Butcher et al, 1974). There is evidence to suggest that baseline pain sensitivity in rats peaks during proestrous and early estrous compared to diestrous (Fillingim and Ness, 2000). Hind paw withdrawal latencies were significantly higher during proestrous than during estrous and diestrous in carrageenan-infammed rats (Tall and Crisp, 2004). In a TMD model of pain, it was reported that females in proestrous exhibited fewer pain behaviors than those in diestrous (Clemente et al, 2004). Modulation of opioid-mediated analgesia by menstrual cycle has also been reported. Opioids are shown to be less potent during estrous compared to females tested in other stages (Craft et al, 2004). There is also some evidence that opioid potency differs between rats tested in ´´early´´ vs ´´late´´ proestrous (Berglund and Simpkins, 1988; Stoffel et al, 2003). Taken together, sex differences and the effect by menstrual cycle suggest that the sex hormones of females may affect behavioral responses to pain. Although the exact mechanisms by which sex hormones affect pain sensitivity, especially in chronic pain conditions, has not been determined.

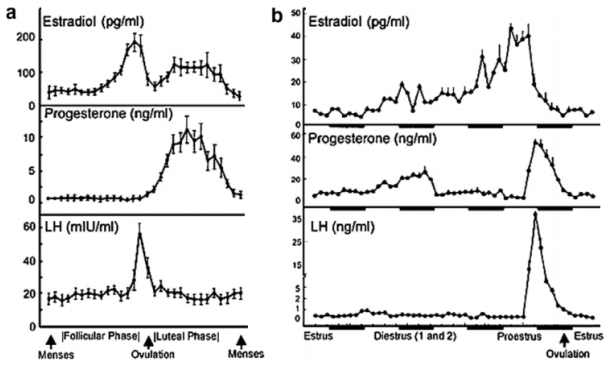

Fig.1 Fluctuation of estradiol, progesterone, and luteinizing hormone (LH) in humans (a) and rats (b) during the menstrual and estrous cycle, respectively (Greenspan et al, 2007).

Generally, the impacts of gonadal steroid hormones fall into two categories, organizational or activational (Cooke et al, 1998). Organizational effects of hormones result in permanent structural and functional differences in the CNS during development. Activational effects are temporary and reversible and result from steroid activation or inhibition on existing circuits in the adult often through specific receptors. That is, hormones continue to promote male or female characteristics in the adult by acting on neural circuits that were organized during development (Aloisi et al, 2006). Animal studies showed that sex differences in pain can often be eliminated by manipulating gonadal hormones during neonatal periods or adulthood. For example, male rodents are more sensitive than females to morphine-induced analgesia (Mogil et al, 2000), whereas neonatally castrated males are less sensitive to morphine than normal males, and in contrast, neonatally androgenized females are more sensitive to morphine than normal females (Cicero et al, 2002; Krzanowska et al, 2002). Gonadectomized male pups exhibited greater mechanical allodynia in carrageenan-induced inflammation and testosterone replacement cannot reverse this phenomenon (Borzan and Fuchs, 2006). LaCroix-Fralish et al. (2005a) demonstrated that female rats developed more hyperalgesia after lumbar nerve injury, whereas the enhanced hypersensitivity in females was reversed in pubertal and adult animals ovariectomized 6 weeks prior to the injury.

1.4 ROLE OF ESTROGEN AND ITS RECEPTORS IN PAIN

In women, rapid decreases in estrogen level at menstruation appear to further increase ongoing muscle and joint pain in TMD and RA as well as in migraine (LeResche et al, 1997; Brandes, 2006). Estrogens are produced primarily in the ovaries during reproductive years (Craft et al, 2004). There are three major natural estrogens in women: estradiol, estrone, and estroil. Estradiol is the predominant form in nonpregnant females whereas estrone is produced during menopause and estriol is the primary estrogen during pregnancy (Ascenzi et al, 2006). In the body estrogens are produced from androgens through aromatization by enzymes. Small amounts of estrogens are also produced by other tissues such as the liver, adrenal glands, and the breasts. These secondary sources of estrogens are especially important in postmenopausal women (Nelson and Bulun, 2001).

Estrodiol is highly lipophylic and therefore can pass the blood–brain barrier and cell membranes. Two types of receptors for estrogen (ERs) have been identified and are

named ER-alpha (ERα) and ER-beta (ERβ). They are intracellular receptors and located in the nucleus (Gruber et al, 2002; Koehler et al, 2005). These receptors are the main mediators of estrogen's effects (Mueller and Korach, 2001). Some estrogen receptors associate with the cell surface membrane and can be rapidly activated by exposure to estrogen (Levin, 2009), but how much this pathway interacts with pain modulation is not clear (Craft, 2007). Estradiol binds equally well to both receptors with high affinity, estrone and estriol bind preferentially to the ERα and ERβ, respectively (Fan et al, 2010). It is generally believed that estrogens diffuse into the cell and bind to the ERs homo- or hetero-dimers to act as transcription factors. Then, this nuclear estrogen-ER complex binds to estrogen response element (ERE) sequences directly or indirectly through protein-protein interactions with activator protein 1 (AP1) site in the promoter region of estrogen-responsive genes, resulting in changes in mRNA levels and protein production and finally, physiological response (Deroo and Korach, 2006).

ERα and ERβ are both present in the DRGs, spinal cord (laminae I and II), trigeminal subnucleus caudalis as well as hypothalamus, amygdala, PAG and dorsal raphe nucleus, areas involved in pain transmission and modulation (Shugrue et al, 1997). ERα and ERβ are differentially regulated in different tissues, including in the CNS (Osterlund et al, 1998) and DRGs (Taleghany et al, 1999). By action on ERα and ERβ, estrodiol modulates nerve growth factor (NGF) receptor in DRGs, endogenous enkephaline expression (Priest et al, 1995) and μ-opioid receptor levels (Amandusson et al, 1996 and 1999; Micevych et al, 1997; Quiñones-Jenab et al, 1997; Micevych and Sinchak, 2001). Animal studies showed that increased formalin-induced paw licking after central administration of estrodial (Aloisi and Ceccarelli, 2000). Estrodial also has been reported to increase pain response after peripheral or spinal cord injury (Gorman et al, 2001; Dina et al, 2007). By large, the role of ERα and ERβ in pain and sex difference in pain has not been thoroughly studied.

1.5 ADENOSINE AND ADENOSINE RECEPTORS IN PAIN

Adenosine is an endogenous nucleoside and a structural component of nucleic acids such as adenosine triphosphate (ATP), adenosine diphosphate (ADP) and cyclic adenosine monophosphate (cAMP) (Fig. 2A). It is a ubiquitous homeostatic substance released from most cells, including neurons and glia under normal conditions or under depolarization, such as during elevated K^+ concentrations, electrical stimulation and glutamate receptor

activation. Adenosine is involved in many biological processes such as energy transfer and signal transduction and also physiological processes such as cardiovascular regulation, cognition, and neuroprotection (Fredholm, 1997; Ribeiro et al, 2002).

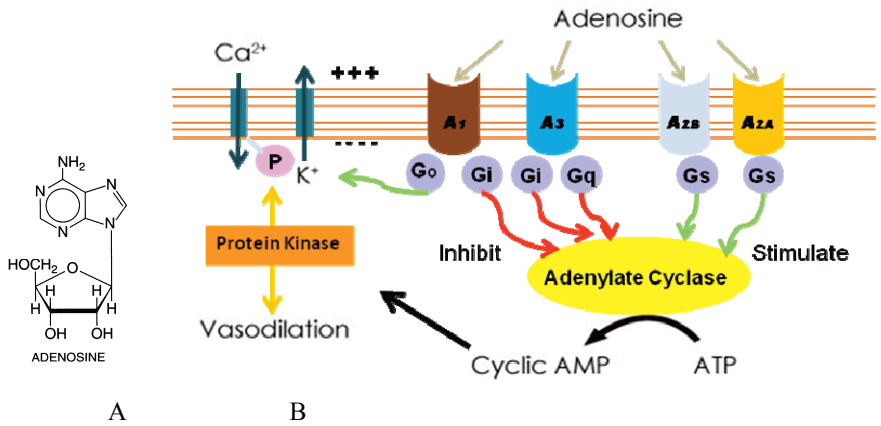

Fig. 2 Structure of adenosine (A) and adenosine receptor coupling (B).

There are at least four subtypes of G-protein coupled receptors, adenosine A_1, A_{2A}, A_{2B} and A_3 (A_1R, $A_{2A}R$, $A_{2B}R$, A_3R) (Fredholm et al, 2001). A_1Rs are usually coupled to inhibitory Gi or Go G-proteins (Linden, 2001). $A_{2A}Rs$ and $A_{2B}Rs$ are coupled to Gs G-protein. A_3Rs are coupled to Gi, Gq, and Go-protein (Fredholm et al, 2001; Ribeiro et al, 2002). $A_{2A}R$ raises cAMP and A_1R decreases cAMP, hyperpolarizes neurons and depresses calcium currents (Dolphin et al, 1986; Dunwiddie and Masino, 2001; Fredholm et al, 2001). A_1R is widely distributed in brain, spinal cord and the periphery (Fredholm et al, 2001), whereas adenosine $A_{2A}Rs$ are predominantly expressed in basal ganglia, in the periphery and in immune cells (Dunwiddie and Masino, 2001).

Adenosine has complex effects on nociceptive pathways and can either enhance or decrease nociception, depending on the site of administration and the receptor subtype activated (Sawynok, 1998; Sawynok and Liu, 2003) (Fig. 2B). Activation of A_1R, located primarily interneurons in the inner lamina II of the superficial dorsal horn (Schulte et al, 2003), primarily produces inhibition of neuronal activity in the spinal cord and DRGs (Dolphin et al, 1986; Li and Peril, 1994; Reeve and Dickenson, 1995; Deuchars et al, 2001) and administration of adenosine agonists that activate A_1Rs produces antinociception in a variety of tests (Sawynok and Liu, 2003). Mice lacking the A_1R have

an enhanced response to nociceptive stimuli under normal conditions, after inflammation and nerve injury (Johansson et al, 2001; Wu et al, 2005) supporting an inhibitory function for A_1Rs in nociception.

The role of A_{2A}Rs in peripheral and spinal nociception is less clear than that of A_1Rs. The A_{2A}R gene is expressed in the DRG and in the peripheral and central presynaptic terminals of sensory afferents in rats (Kaelin-Lang et al, 1998), suggesting the potential involvement of A_{2A}R in pain. A_{2A}R knock-out ($A_{2A}R^{-/-}$) mice are hypoalgesic in tests of acute nociception (Ledent et al, 1997) and the selective A_{2A}R antagonist SCH 58261 attenuates nociceptive responses in acute pain tests and in the formalin test in mice, suggesting a pronociceptive role of A_{2A}Rs (Godfrey et al, 2006; Hussey et al, 2007). In contrast, CGS 21680, a selective A_{2A}R agonist, has also been shown to produce antinociception upon intrathecal administration in the formalin test and after inflammation (Poon and Sawynok, 1998; Yoon et al, 2005).

Sex difference in adenosine modulation of physiological functions has also been reported. For example, the magnitude of the involvement of the A_1Rs in regulation of heart rate, body temperature and locomotor activity in mice is different in males and females as studied in knock-out mice (Yang et al, 2007). The neurotoxic effects of A_1R antagonism during ethanol withdrawal also exhibit sex difference (Bulter et al, 2008 and 2009) as does the incidence of chest pain during adenosine infusion in human cardiac patients (Kumar and Movahed, 2002). However, the role of A_{2A}R in sex difference in pain has not been examined.

1.6 ANIMAL PAIN MODELS AND GENETICALLY MODIFIED MICE

Animal models for pain can be roughly categorized into three groups, tests of nociception, models of acute pain and chronic pain. The nociceptive pain tests evaluate the response of animals to nociceptive stimuli using for example the hot plate, tail flick or paw pressure tests. Acute pain models include cutaneous inflammation (carrageenan or Freund's adjuvant), monoarthritis (carrageenan, kaolin), traumatic injury (skin incision, bone fracture) and some models of visceral pain (Ness and Gebhart, 1988). There are also animal models for chronic pain, including models for cancer pain, polyarthritis and neuropathic pain. In recent years, a large number of neuropathic pain models have been developed after various injuries to the peripheral or central nervous system. The rat sciatic

nerve as well as its branches has been used most often for the study of peripheral neuropathy because they innervate the hind paw, which is a suitable site for sensory testing (Bennett and Xie, 1988; Seltzer et al, 1990; Kim and Chung, 1992; Hogan, 2002). Models using other forms of injury (such as ischemia, toxin or viral infection) or affecting other peripheral nerves (such as infraorbital nerve innervating facial region) have also been developed (Xu and Wiesenfels-Hallin, 2003). There are also central neuropathic pain models, mostly after injury to the spinal cord using the photochemically-induced ischemia technique (Hao et al, 1991), compression (Martin et al, 1992; von Euler et al, 1997), weight drop (Siddall et al, 1995), and surgical hemisection (Christensen et al, 1996).

In the current work, several pain tests and models were used. These include nociceptive tests, carrageenan-induced acute inflammation, photochemically-induced sciatic nerve injury (Hao et al, 2000), infraorbital nerve injury (Dominguez et al, 2009) and spinal cord injury models (Xu et al, 1992).

Transgenic knock-out mice have been widely employed in pain research (Lariviere et al, 2001; Malmberg and Zeitz, 2004; LaCroix-Fralish and Mogil, 2009) and yielded a great deal of knowledge (Mogil and McCarson, 2000). By using knock-out mice, the physiological importance of certain genes can be explored for their involvement in pain either during development or in adults. For examples, knock-out studies have established important roles for neurotrophins and their receptors such as NGF (Crowley et al, 1994) and TrkA (Smeyne et al, 1994) in the development of nociceptors. Null mutations in cytokine genes have shown a role for interleukin (IL)-6 (Xu et al, 1997) and interferon (IFN)-α (Robertson et al, 1997) in the development of inflammation and neuropathic pain, respectively. Furthermore, the physiological and pharmacological contribution of receptors can be identified or verified with target deletion of receptors which are potentially associated with pain such as in the case of the opioid system. Results gained from opioid receptor knock-out mice showed that each receptor (μ, δ or κ-opioid receptor) was implicated in different acute pain modalities in a distinct manner and to a different extent, which is in line with pharmacological data (Kieffer and Gavériaux-Ruff, 2002). Thus, using knock-out mice together with additional pharmacological agents could contribute to a better understanding of pain mechanisms. In this thesis, I used ERα knock-out ($ER\alpha^{-/-}$), ERβ knock-out ($ER\beta^{-/-}$) and $A_{2A}R^{-/-}$ mice to elucidate their roles in the sex difference in pain.

2 AIMS OF THE THESIS

The general aim of the work presented in this thesis is to study sex differences in experimental models of acute and chronic pain. In particular, we want to study

1. The role of estrogen receptors in sex differences in mechanical nociception, inflammation and in the development of neuropathic pain using knock-out mice
2. To study the role of $A_{2A}R$ in nociception and inflammatory hyperalgesia using knock-out mice
3. To determine sex differences in the development of acute pain-like behaviors in rats after spinal cord injury and to study the impact of estrous cycle on pain development
4. To determine sex differences in the development of localized and spread mechanical hypersensitivity in rats after infraorbital nerve injury and to study the impact of estrous cycle on pain development

3 MATERIALS AND METHODS

3.1 ANIMALS

All experiments were conducted according to the Ethical Guidelines of IASP and were approved by the local research ethics committee (Stockholm Södra Försöksdjursetiska Nämd). All animals (rats and mice) were housed in standard lab conditions (22 °C; 12 h light/dark cycle, lights on at 6 a.m.) with access to food and water *ad libitum*. In paper I, the ER$\alpha^{-/-}$ and wild-type C57BL/6 mice were from Taconic M&B (Eiby, Denmark). The ER$\beta^{-/-}$ and wild-type C57BL/6 mice were generated and supplied by the Department of Biosciences and Nutrition, Karolinska Institutet. In paper II, the $A_{2A}R^{-/-}$ mice were generated and bred in the Department of Physiology and Pharmacology, Karolinska Institutet and the wild-type C57BL/6 mice were from Charles River (Sollentuna, Sweden). In papers III and IV, male and female Sprague-Dawley rats (Mollegård Ltd., Skensved, Denmark) were used.

3.2 GENERATION OF ESTROGEN RECEPTOR KNOCK-OUT MICE

The methods for generating ER$\alpha^{-/-}$ and ER$\beta^{-/-}$ mice have been described previously (Lubahn et al, 1993; Krege et al, 1998). The targeted embryonic stem (ES) cells are from 129/J and 129P2/OlaHsd strains, respectively, and the resultant chimeras were backcrossed to C57/BL/6 for 10 or 8 generations before homozygous ER$\alpha^{-/-}$ and ER$\beta^{-/-}$ mice were generated using heterozygotes.

3.3 GENERATION OF ADENOSINE A_{2A} RECEPTOR KNOCK-OUT MICE

The generation and breeding of the adenosine $A_{2A}R^{-/-}$ mice was described previously (Chen et al, 1999; Halldner et al, 2004). Briefly, the mouse $A_{2A}R$ gene was cloned from mouse 129-Steel genomic library. The second coding exon was inactivated in ES cells with a standard replacement-type vector containing positive selection marker (PGK-*Neo* cassette). The correct integration of the mutant allele was demonstrated by Southern blotting, using a non-overlapping 3' probe after digestion with *Bam*HI (a restriction enzyme). One of the ES cell clones with the mutant allele was used to generate chimaeric mice. The chimaeric mice were bred with C57BL/6 to produce heterozygous genotype ($A_{2A}R^{+/-}$). Such mice were intercrossed to generate homozygous ($A_{2A}R^{-/-}$), heterozygous ($A_{2A}R^{+/-}$) and wild-type littermates (Chen et al, 1999). $A_{2A}R^{-/-}$ mice was made on mixed (129-Steel x C57BL/6) genetic background, and were backcrossed for more than 10 generations with C57BL/6 to achieve essentially pure congenic lines**.**

3.4 CARRAGEENAN-INDUCED INFLAMMATION IN MICE

Mice were anaesthetized with chloral hydrate (300 mg/kg, i.p.). λ-carrageenan (Sigma-Aldrich) at a concentration of 2% dissolved in 20 µl saline or 20 µl normal saline was injected subcutaneously (s.c.) into the plantar area of the left or right hind paw, respectively. The effect of carrageenan or saline on the response of the mice to mechanical stimuli was determined 24 h after the injection. Paw thickness was measured with a caliper at metatarsal level before and 24 h after the injection.

3.5 PHOTOCHEMICALLY-INDUCED INJURY TO NERVES AND SPINAL CORD

3.5.1 Sciatic nerve injury in mice

Mice were anesthetized with chloral hydrate. The common sciatic nerve was exposed at midthigh level and gently from the surrounding tissue over a distance of about 1 cm proximal to the trifurcation. After being injected intravenously (i.v.) with erythrosine B (Aldrich, 32,5mg/kg) via the tail vein, the exposed nerve was irradiated with a tunable argon laser (Innova model 70, Coherent Laser Products Division, Palo Alto, CA, USA) operating at 514 nm with an average power of 0.16 W. The interaction between the dye and the laser beam causes vascular occlusion and focal ischemia (Gazelius et al, 1996; Kupers et al, 1998; Hao, et al, 2000). The irradiation time for mice was 45 seconds. During the period of irradiation, a heating pad was used to maintain the body temperature between 37-38 °C in the mice. After irradiation the wound was closed in layers and the animals were returned to their home cages.

3.5.2 Spinal cord injury in rats

Under chloral hydrate anesthesia, a midline incision was made on skin overlying T12-L1 vertebral segments. The rats were placed under the laser beam and irradiated as above at vertebral segment T13 (spinal cord level L4-5) for 5 or 10 min. Immediately before irradiation erythrosine B was injected i.v. via tail vein and the injection was repeated after 5 min. After irradiation, the wound was closed and the rats were put back to their cages. During the period of irradiation, a heating pad was used to maintain the body temperature between 37-38 °C in rats.

3.5.3 Infraorbital nerve injury in rats

Under chloral hydrate anesthesia the left infraorbital nerve was exposed via a longitudinal incision at the maxillary region. The different branches of the infraorbital nerve were held together and lifted with a glass hook while a piece of aluminium foil was placed under the nerve. The nerve was irradiated as above for 6 min. Immediately before irradiation erythrosine B was injected i.v. via tail vein and the injection was repeated after 5 min. After irradiation the wound was closed in layers and the rats were returned to their home cages for recovery. During the period of irradiation, a heating pad was used to maintain the body temperature between 37-38 °C in rats.

3.6 BEHAVIORAL TESTS OF NOCICEPTION

3.6.1 Tests of hindpaw sensitivity in mice to mechanical stimulation

The mice were placed in plastic cages with a metal mesh floor. The plantar surface of the hindpaw was stimulated with a set of calibrated monofilaments (von Frey hairs, Stoelting, IL, USA) with increasing force until the animal withdrew the limb. The range of stimulus forces was from 0.008 g to 10 g. Each monofilament was applied five times. The withdrawal threshold was taken as the force at which the animal withdrew the paw from at least three of five consecutive stimuli. The experimenters were blind to the genetic status of the mice.

3.6.2 Tests of acute mechanical hypersensitivity after spinal cord injury in rats

Vocalization thresholds to graded mechanical touch/pressure on the back of the rats were tested. During testing the rats were gently restrained in a standing position and the von Frey hair was pushed onto the skin in dermatomes rostral to the irradiated spinal segment-the upper or lower back area. The frequency of stimulation was about 1/s; and at each intensity the stimuli were applied 5-10 times. The intensity of stimulation which induced consistent vocalization (>75% response rate) was considered as pain threshold. The cut-off value was a von-Frey hair with 100g force.

3.6.3 Motor deficits after spinal cord injury

Motor deficits after spinal cord injury were evaluated using a combined neurological score using four different tests (Table. 1): motor score (observation of walking in an open field), toe spread (spreading of toes when lifted), righting reflex and extension

withdrawal (reflex replacement of hind leg when tested). A normal rat has a score of 0 whereas a completely paralyzed rat has a score of 70 (Hao et al, 1996).

Table. 1 Neurological score for evaluation of motor function of rats.

Grade	Description	Points
Motor score		
0	Normal walking	0
1	Walks with only mild deficit	5
2	Hindlimb can support weight	15
3	Frequent movement of hindlimb, no weight bearing	25
4	Minor movement in hindlimb, no weight bearing	40
5	No movement of hindlimb, no weight bearing	45
Toe spread		
0	Normal full toe spread	0
1	Partial spreading of toes	2,5
2	No spreading of toes	5
Righting		
0	Normal righting counter to direction of the roll	0
1	Weakened attempt to right	5
2	Delayed to right itself	10
3	No attempt to right	15
Extension withdrawal		
0	Normal	0
1	Weak and slow reflex to withdraw the hindlimb	2,5
2	No withdraw reflex	5

3.6.4 Assessment of localized and spread mechanical sensitivity after infraorbital nerve injury

The von Frey filaments were applied in ascending order to the territory of the infraorbital nerve on the hairy skin of the vibrissal pad bilaterally. The threshold was taken as the force at which the rat responded by withdrawal, escape, or attack in 3 of 5 stimuli.

The response to mechanical stimulation of skin areas of the neck, upper and lower back, and flanks was also tested. During testing, the rats were gently restrained in a standing position, and the von Frey filament was pushed onto the skin until the filament became bent. The frequency of stimulation was 1/s, and at each intensity, 5 to 10 stimuli were applied. The intensity of stimulation, which induced consistent vocalization at >75% response rate, was considered a pain threshold.

3.7 DETERMINATION OF THE ESTROUS CYCLE IN RATS

Vaginal smears were performed to ascertain which phase of the estrous cycle the female rats were in at the time of irradiation. The female rats were held vertically with one hand and the other hand inserted a micropipette 0.3 mm into the vaginal canal. The vagina was flushed three times with 10 µl 0.9% saline solution. The solution was put on a microscope slide and a cover glass was placed on it and examined under a light microscope. The smears were taken and evaluated 09:00-11:00 since the different estrous stages are more pronounced in the morning (Krinke, 2000). No dyes were applied since it has been shown that it does not significantly influence the evaluation (Marcondes et al, 2002).

3.8 MORPHOLOGICAL ASSESSMENT OF SPINAL CORD INJURY IN RATS

Morphology was performed 30 days after spinal cord injury. Rats were deeply anesthetized with chloral hydrate and perfused with phosphate buffer saline (PBS). Spinal segment L3-6 containing the irradiated area was removed and frozen immediately at -70°C. Transverse sections (14 µm thick) from the spinal cord were air dried, stained with hematoxylin and examined under a light microscope. Images were taken with a Nikon DXM 1200 digital camera. The epicenter of the injury was determined with an injury severity score. The percentage of the injured area in the epicenter was analyzed with the Easy Image software (version 3000). The length of the injury was also calculated as the distance from the beginning slice of the injury to the end slice of the injury.

3.9 IMMUNOCHEMISTRY IN $A_{2A}R^{-/-}$ MICE

Wild-type and $A_{2A}R^{-/-}$ mice were anesthetized with pentobarbital intraperitoneally (i.p.) and transcardially perfused with 4% paraformaldehyde in PBS. Brains and DRG (L4/5) were dissected and cryoprotected in PBS, 30% sucrose and NaN_3 at 4ºC overnight. Tissues were sectioned on a cryostat (brain 35 µm; DRG 14 µm) and staining was performed either with free-floating brain sections or with DRG sections mounted on chrome-alum-coated slides. Sections were washed two times in PBS at room temperature and blocked for 1.5 h in PBTA (PBS, 0.25% Triton X-100, 3% BSA, 0.01% NaN_3). Primary antibody (1: 200; Santa Cruz goat IgG anti A_{2A} sc-7504 (R18)) was incubated at 4ºC overnight. Following 3 washes in PBS, secondary antibody (1:500, Jackson Immunoresearch, Cy2-coupled donkey anti goat) was applied for 1.5 h at room temperature. After extensive washing in PBS, sections were air-dried on glass slides

overnight and coverslipped with DAKO fluorescent mounting medium prior to analysis on a Zeiss LSM510 META laser scanning microscope.

3.10 REAL TIME PCR ANALYSIS IN $A_{2A}R^{-/-}$ MICE

DRGs were dissected out from wild-type and $A_{2A}R^{-/-}$ mice and total RNA was isolated using the Totally RNA kit (Ambion) according to the manufacturer's instructions. The RNA was eluted in RNAse free water. Further quality control before the microarray analysis was performed on an Agilent 21000 Bioanalyser (Agilent Technologies). cDNA synthesis was performed with the GeneAmp RNA PCR kit (Applied Biosystems) using random hexamers and the MuLV reverse transcriptase according to the manufacturer's instructions. The adenosine A_1, A_{2A}, A_{2B}, and A_3 receptor mRNA in the DRG was detected by using RT-PCR (Halldner et al, 2004). Primers and probes that were used are described elsewhere (Chunn et al, 2001). The RT-PCR reactions were performed in triplicate with the TaqMan Universal PCR master mix in an ABI Prism 7000 Sequence Detector System (Applied Biosystems, Foster City, CA). Values are expressed as the difference in the number of cycles needed to reach the detection threshold (ct = cycle at threshold) using β-actin as a reference ($\Delta ct = ct_{\text{adenosine receptor}} - ct_{\beta\text{-actin}}$).

3.11 DRUGS AND STATISTICS

The following drugs were used: λ-carrageenan in papers I and II, the selective $A_{2A}R$ agonist CGS21680 and the selective $A_{2A}R$ antagonist ZM-241,385 in paper II. All the drugs were from Sigma-Aldrich. λ-carrageenan and CGS21680 were dissolved in 0.9% saline. ZM-241,385 was dissolved in DMSO and then further diluted in cremophor EL and saline.

Data are presented as median ± median absolute deviation (MAD) or mean ± standard error of the mean (SE.M). where appropriate. The data were analyzed with the Mann–Whitney *U*-test, Wilcoxon Signed rank test, ANOVA with repeated measures followed by Fisher's PLSD test as well as paired- or unpaired *t*-test. All statistics were made with StatView on a PC computer.

4 RESULTS

4.1 ESTROGEN RECEPTORS IN SEX DIFFERENCE IN PAIN (PAPER I)

In this study, we examined the role of estrogen receptors ERα and ERβ in sex difference in pain using $ER\alpha^{-/-}$ and $ER\beta^{-/-}$ mice and their wild-type controls.

4.1.1 Response to mechanical stimulation under normal condition

A sex difference in withdrawal threshold to mechanical stimulation was observed in wild-type mice for both $ER\alpha^{-/-}$ and $ER\beta^{-/-}$ mice with females exhibiting significantly lower response threshold than males (Fig. 1, paper I). No sex differences was seen in the $ER\alpha^{-/-}$ and $ER\beta^{-/-}$ mice in that the females exhibited elevated threshold, which was comparable to males (Fig. 1, paper I). The withdrawal threshold to mechanical stimulation in the male mice was similar between the knock-outs and wild-types (Fig. 1, paper I).

4.1.2 Carrageenan-induced inflammation in $ER\alpha^{-/-}$ and $ER\beta^{-/-}$ mice

Twenty-four hours after injection of carrgeenan in a hindpaw, the injected paw exhibited local edema in wild-type mice with no difference between the sexes (Fig. 2, paper I). There was also no significant difference in the extent of paw edema between the wild-types and either $ER\alpha^{-/-}$ or $ER\beta^{-/-}$ mice (Fig. 2, paper I).

All mice developed hypersensitivity to mechanical stimulation. The paw withdrawal threshold was significantly lower in female than in male mice 24 h after inflammation in both strains of wild-types (Fig 3, paper I). Again, no sex difference was observed in mice lacking either type of estrogen receptors, with $ER\alpha^{-/-}$ and $ER\beta^{-/-}$ females reacting similarly to males (Fig 3, paper I).

4.1.3 Photochemically-induced sciatic nerve injury in $ER\alpha^{-/-}$ and $ER\beta^{-/-}$ mice

Photochemically-induced sciatic nerve partial injury was generated in $ER\alpha^{-/-}$ and $ER\beta^{-/-}$ mice and their wild-type controls. All mice developed mechanical hypersensitivity of the hind paw. There were no sex differences in the extent of hypersensitivity in the two wild-type strains (Fig. 4, paper I). There were also no differences between the knock-outs and their respective wild-type controls during the 28-day observation period (Fig. 4, Paper I).

4.2 THE ROLE OF ADENOSINE $A_{2A}R$ IN CARRAGEENAN-INDUCED MECHANICAL HYPERALGESIA AND SEX DIFFERENCE IN MICE (PAPER II)

In this study, we examined the role of $A_{2A}R$ in carrageenan-induced hyperalgesia and in sex differences to the effects of a selective $A_{2A}R$ agonist and antagonist using $A_{2A}R^{-/-}$ mice and their wild-type controls.

4.2.1 Reduced hyperalgesia in $A_{2A}R^{-/-}$ mice after inflammation

$A_{2A}R^{-/-}$ mice exhibited similar baseline mechanical paw withdrawal thresholds as did age and weight matched wild-type mice of both sexes (Fig. 1A, paper II). Both wild-type and $A_{2A}R^{-/-}$ mice developed mechanical hypersensitivity 24 h after s.c. carrageenan (Fig. 1A, paper II). There was no significant sex difference in either wild-types or $A_{2A}R^{-/-}$ mice in baseline paw withdrawal thresholds and carrageenan-induced hyperlagesia (not shown). Although a substantial amount of hyperalgesia was also observed in $A_{2A}R^{-/-}$ mice, the difference between the $A_{2A}R^{-/-}$ mice and wild-types was significant in that the wild-types developed more severe hyperalgesia than the $A_{2A}R^{-/-}$ mice. Carrageenan also produced significant paw edema 24 h after injection in both wild-types and $A_{2A}R^{-/-}$ mice with no significant differences between the groups (Fig. 1B, paper II).

4.2.2 The effect of the $A_{2A}R$ antagonist ZM-241,385 on inflammatory hyperalgesia

The effect of ZM-241,385 was examined in separate groups of male and female wild-type mice. Twenty-four hours after s.c. carrageenan, as mentioned above, the wild-type mice developed mechanical hypersensitivity (Fig. 2, paper II). S.c. ZM-241,385 injected directly into the hindpaw significantly increased the paw withdrawal threshold to mechanical stimulation in female, but not male, mice. Vehicle produced no significant effect in male and female mice. Comparing to the vehicle group, ZM-241,385 showed significant antinociceptive effect in both males and females, but the effect was significantly larger in females than males (Fig. 2, paper II).

4.2.3 The effect of the $A_{2A}R$ agonist CGS 21680 on paw withdrawal threshold

Ten µl s.c. saline injected into the paw produced a modest, but significant, decrease in paw withdrawal threshold for 60 min in wild-type mice (Fig. 3A, paper II). S.c. injection of CGS 21680 in the paw at 1 nmol/10 µl produced a more profound and persistent decrease in paw withdrawal threshold in the $A_{2A}R^{-/-}$ mice and in the wild-types than saline (Fig. 3A, paper II). The reduction in paw withdrawal threshold was significantly less in the $A_{2A}R^{-/-}$ mice than in the wild-types (Fig. 3A, paper II). In the wild-type mice, the effect of CGS 21680 was significantly longer lasting in females than in males, a difference that was not seen in the $A_{2A}R^{-/-}$ group (Fig. 3B, paper II). S.c. CGS 21680 injected into the hindpaw did not produce paw edema in any of the groups (data not shown). Injection of 1 nmol CGS 21680 s.c. in the neck region did not affect mechanical sensitivity of hindpaws in either male or female wild-type mice (Fig. 3 A, B, paper II).

4.2.4 Low level of $A_{2A}R$ expression in DRG

In order to confirm that $A_{2A}R$ are present in mouse DRG, which have been reported in rats (Kaelin-Lang et al., 1998), we examined its presence using immunohistochemistry employing a polyclonal goat anti-$A_{2A}R$ antibody (Fig. 4A, paper II). However, even though A_{2A}-like immunoreactivity was readily detectable in the caudate putamen (CP), where $A_{2A}R$ expression is high, it was hardly detectable in either DRG (Fig. 4A, paper II) or skin (not shown). Importantly, the weak fluorescence was similar in DRG of $A_{2A}R^{-/-}$ compare to wild-type mice. This was even more evident in skin samples from the mouse paw, where background immunoreactivity was comparable in wild-type and $A_{2A}R^{-/-}$ mice (not shown). In contrast, the prominent A_{2A}–like immunoreactivity detected in mouse caudate putamen was eliminated in the mice lacking the adenosine $A_{2A}R$ (Fig. 4A, right panel, paper II), underlining the specificity of the antibody. Thus, from the immunohistochemistry data, we conclude that the expression levels of $A_{2A}R$ in DRG is much lower than in the CP. Employing real-time PCR, on the other hand, we found that $A_{2A}R$ mRNA is detected in mouse DRG to an extent similar to $A_{2B}R$ mRNA, but far less than A_1R mRNA. A_3R mRNA was almost undetectable (Fig. 4B, paper II). Comparable real-time PCR studies using cDNA from mouse CP resulted in a $2^{-\Delta ct}$ value for $A_{2A}R$ expression that was 0.096 (i.e. about two orders of magnitude higher than in DRG) supporting our findings from the immunohistochemistry on lower levels of $A_{2A}R$ expression in the DRG.

4.3 SEX DIFFERENCES IN THE DEVELOPMENT OF ALLODYNIA IN RATS AFTER SPINAL CORD INJURY (PAPER III)

In this study, we assessed sex differences in the development of acute allodynia in rats after photochemically-induced spinal cord injury and the impact of the stage of the estrous cycle at the time of injury.

4.3.1 Sex differences in acute allodynia

The vocalization threshold to mechanical stimulation of the flank and upper back area in normal rats was 80-100 g with no significant difference between males and females. Within 1 day after spinal cord irradiation for 5 or 10 min, female rats showed markedly decreased response threshold to mechanical stimulation in these regions (Fig. 1A, B, paper III). In contrast, there was no significant decrease in mechanical response threshold in male rats after 5 min irradiation (Fig. 1A, paper III). After 10 min irradiation, male rats also showed significant decrease in vocalization threshold (Fig. 1B, paper III). There was a significant sex difference between males and females after 10 min irradiation with females having lower threshold and slower recovery (Fig. 1B, paper III).

4.3.2 No impact of estrous cycle

Estrous stage was controlled in female rats at the time of the spinal cord injury and no significant differences were observed in baseline vocalization threshold and mechanical hypersensitivity after spinal cord injury among three groups of female rats irradiated at different stages of the estrous cycle (Fig. 2, paper III).

4.3.3 Sex difference in acute allodynia is not due to difference in the extent of injury

Motor deficits were evaluated and morphological analysis was conducted after spinal cord injury to determine if sex difference after spinal cord injury was due to different magnitude of injury in male and female rats. Both male and female rats developed moderate neurological motor deficits after 5 or 10 min spinal cord irradiation. There was no significant difference in combined neurological scores between males and females (Fig. 3A, Paper III). Morphological comparison was made in rats after 10 min spinal cord

irradiation which produced moderate injury to dorsal spinal cord (Fig. 3B, Paper III). No significant differences were observed between males and females in the magnitude of the injured area in the epicenter or to the length of injury (Fig.4, Paper III).

4.4 SEX DIFFERENCES IN THE DEVELOPMENT OF ALLODYNIA IN RATS AFTER INFRAORBITAL NERVE INJURY (PAPER IV)

In this study, we assessed sex differences in the development of localized and spread mechanical allodynia in rats after infraorbital nerve injury and the impact of estrous cycle at the time of and after injury.

4.4.1 Baseline response threshold to mechanical stimulation

A significant difference in baseline threshold to mechanical stimulation was found between male and female rats both in the facial and at the upper flank region (Fig. 1A, paper IV) with normal females having significantly lower threshold on the face and upper flank region compared to males. Females tested at different stages of estrous cycle did not show significant difference in baseline response threshold on either the face or upper flank (Fig. 1A, B, paper IV).

4.4.2 Sex difference in the development of localized and spread allodynia

After infraorbital nerve injury, both males and females developed mechanical hypersensitivity on the ipsilateral face and upper flank and back region. The hypersensitivity was significantly more severe and prolonged in females (Fig. 2A, B, paper IV). ANOVA with repeated measurement indicated a significant overall difference between the two sexes during the observation period and there was an interaction between sex and time (Fig. 2A, B, paper IV).

4.4.3 Lack of impact by estrous stage on mechanical hypersensitivity after infraorbital nerve injury

When the females were divided into the three different estrous stages at the time of irradiation, there was no significant difference in the pattern of hypersensitivity development on the face and upper flank among the groups (Fig. 3A, B, paper IV). During weeks 1-3 and 12-14 after infraorbital nerve injury, hypersensitivity on the face and flank were tested daily for 15 days to cover 3 continuous estrous cycles. Comparison

was made among each estrous stage both in face and upper flank region. After injury, only 10% of the females exhibited regular cycles (data not shown). However, it was still possible to identify various estrous stages through vaginal smear. No significant difference of mechanical hypersensitivity was found in the face or upper flank region across the estrous stages (Fig. 4A, B, paper IV). The hypersensitivity on the face was significantly less during weeks 12-14 compared to weeks 1-3 after the injury. In contrast, the hypersensitivity on the flanks during weeks 12-14 was similar to weeks 1-3, suggesting spread hypersensitivity was more persistent than localized hypersensitivity.

5 DISCUSSION

5.1 SEX DIFFERENCE IN PAIN SENSITIVITY IN RODENTS

Sex difference in nociceptive response in rodents, with females generally being more sensitive, has been shown in previous studies using several nociceptive assays, including the von Frey test as used in our studies (Mogil et al, 2000; Chesler et al, 2002; Chillingworth et al, 2006). In paper I, such difference again was demonstrated in both strains of wild-type mice for estrogen receptor knock-out mice in baseline paw withdrawal threshold to mechanical stimulation. In paper II, however, we did not observe a significant sex difference in baseline mechanical pain threshold in wild-type mice and $A_{2A}R^{-/-}$ mice. Similarly we also noted that in Sprague-Dawley rats, sex difference was not readily observed for basal mechanical pain threshold. In study IV, there was a significant sex differences for response threshold for mechanical stimulation on the face and upper back region whereas in study III, such difference was not significant for the trunk and lower back region. Influence of site of testing on determination of sex difference has also been previously noted in our and other laboratories (Pajot et al, 2003; Dominguze et al, 2009). It is thus suggested that although sex difference in basal mechanical pain threshold can be demonstrated in rodent, the magnitude of such difference is moderate and subjected to influence by many factors. One of such factor is genetic as it has been shown that sex difference in pain responses in rodents has been shown to be strain dependent (Kest et al, 2000; Deleo and Rutkowski, 2000; LaCroix-Fralish, 2005a) which may explain the lack of sex difference observed in wild-types for $A_{2A}R^{-/-}$ mice as it has different genetic background than the wild-types for $ER\alpha^{-/-}$ and $ER\beta^{-/-}$ mice.

In study I, we also observed that after carrgeenan-induced inflammation, the paw withdrawal threshold in female mice was significantly lower than that in males while in study II, the sex difference did not reach significance after inflammation. This may also be explained by genetic differences between the strains. Another point which needs to be considered is that since there is a sex difference in baseline paw withdrawal threshold with females having low threshold, the relative change after inflammation may not show sex difference. However, we consider it is more relevant to consider the actual threshold after inflammation to determine sex difference as in a clinical setting, pain sensitivity before diseases is not usually considered as a factor.

5.2 THE INVOVEMENT OF ERα AND ERβ IN SEX DIFFERENCE IN PAIN

As mentioned in paper I, sex difference was demonstrated in both strains of wild-type mice in baseline to mechanical stimulation. Male ERα$^{-/-}$ and ERβ$^{-/-}$ had similar baseline mechanical response threshold as their wild-type controls. However, female ERα$^{-/-}$ and ERβ$^{-/-}$ exhibited significantly elevated response threshold, comparable to males, under normal conditions. Thus, the sex difference observed in baseline between wild-type males and females was eliminated in the knock-outs, suggesting a role of ERα or ERβ in determining sex difference in baseline nociception.

The same direction of sex difference in carrageenan-induced mechanical hyperlagesia was also observed in wild-type mice. Both male and female wild-type controls developed similar extent of paw edema, suggesting that the increased mechanical hypersensitivity in females does not seem to be caused by an increased inflammatory response. Again, such sex difference was eliminated in ERα$^{-/-}$ and ERβ$^{-/-}$. Our results agree to some extent with those of Spooner et al. (2007) in which female ERβ$^{-/-}$ mice showed reduced response in the formalin test compared to wild-type controls. These authors did not find that ERβ ablation influenced hot plate latency in females, although there was a sex difference with females being more sensitive than males. This difference from our results may be due to the difference in stimulation and tests used. The lack of phenotypic changes in the male knock-outs suggests that the difference seen in the females does not reflect a generalized action of estrogen or differences in background genes, but rather a sex-specific role of estrogen in mechanical nociception and important in determining sex differences.

No sex difference was seen in mechanical hypersensitivity after partial sciatic nerve injury in wild-type or knock-out mice. This is similar to our previous results in rats (Dominguez et al, 2009). Sex difference in the development of neuropathic pain-like behaviors after nerve injury in rodents has not been consistently reported and may be related to the strains of animal used and experimental models (Deleo and Rutkowski, 2000; LaCroix-Fralish et al, 2005b). The lack of difference between the knock-out male and females suggests again that the role of estrogen receptors in mechanical nociception is sex-specific and important in determining sex differences. It should be noted that in the clinical setting sex differences are observed in the prevalence of many neuropathic pain conditions (Greenspan et al, 2007).

It is not clear which estrogen and its receptors mediate sex difference in mechanical nociception in mice. We also do not know whether the two estrogen receptors play the same or different roles in this process, despite similar phenotypes. Exogenously applied estrogen has been shown to increase pain response in rodents, although the effect is not sex-specific (Aloisi and Ceccarelli, 2000; Craft et al, 2004). The increase in mechanical response threshold in female knock-outs may be due to the removal of an ongoing activational excitatory effect by estrogen in adult female mice. Estrogen receptors have different functions during neurogenesis. ERα activation induces increased length and number of neurites, whereas ERβ activation modulates only neurite length (Papka et al, 2003). ERβ is also essential for morphogenesis and maintenance of the spinal dorsal horn interneurons (Fan et al, 2007). Thus, the phenotype observed in the female knock-outs may also be due to the developmental changes in female mice brought about by the organizational effect of estrogen during development.

5.3 $A_{2A}R$ ACTIVATION IN INFLAMMATORY HYPERALGESIA AND SEX DIFFERENCES IN PAIN

We observed that $A_{2A}R^{-/-}$ mice and their wild-type controls displayed similar baseline nociceptive response to mechanical stimulation, which is in contrast to the $A_1R^{-/-}$ mice that showed reduced mechanical threshold under normal conditions in a previous study (Johansson et al, 2001; Wu et al, 2005). This suggests that unlike the A_1R, the $A_{2A}R$ may not be involved in determining mechanical pain threshold under normal conditions.

After inflammation, both $A_{2A}R^{-/-}$ and wild-type mice developed mechanical hypersensitivity after 24 h that was associated with paw edema. The magnitude of hypersensitivity was significantly reduced in $A_{2A}R^{-/-}$ mice compared to wild-types. The extent of paw edema was similar between $A_{2A}R^{-/-}$ and the wild-type mice, indicating that the reduction in mechanical hypersensitivity in the $A_{2A}R^{-/-}$ mice is not directly related to the extent of peripheral inflammation. In a previous study, we observed that $A_3R^{-/-}$ mice exhibited a reduction of inflammatory hyperalgesia to heat stimulation, which was related to the extent of inflammation in the paw (Wu et al, 2002).

Although no sex difference was found in inflammatory hyperalgesia and paw edema in wild-type or $A_{2A}R^{-/-}$ mice, a pharmacological sex difference were found in response to an

$A_{2A}R$ agonist and antagonist. The selective $A_{2A}R$ antagonist ZM-241,385 reduced inflammatory hypersensitivity upon direct local injection, which was more effective in females. In line with this observation, we found local injection of the $A_{2A}R$ agonist CGS 21680 produced a hyperalgesic effect, which was again more profound in the wild-type females than in males. This indicates that the $A_{2A}R$ is involved in the inflammatory hyperalgesic response to carrageenan and a sex difference in response to $A_{2A}R$ activation in the periphery. However, sex differences in response to $A_{2A}R$ activation did not appear to be sufficiently large to impact sex differences in inflammatory hypersensitivity in the present study.

We do not know which type of cell(s) that is responsible for the effect of $A_{2A}R$ activation on pain sensitivity. It is well known that $A_{2A}Rs$ are present on many types of cells in the immune system (Fredholm et al, 2001). The present data confirm that cells in the DRG, perhaps neurons, express $A_{2A}R$ mRNA, and hence probably the receptor. The expression level is, however, not high. Despite this uncertainty the results suggest that peripheral actions of $A_{2A}R$ antagonists may be relevant in controlling some types of pain. Caffeine (and its metabolites theophylline and paraxanthine) are $A_{2A}R$ antagonists, and caffeine is a known additive in several pain medications (Sawynok, 1998).

5.4 SEX DIFFERENCE IN THE DEVELOPMENT OF NEUROPATHIC PAIN-LIKE BEHAVIORS IN RATS

We conducted two neuropathic-pain models in rats with ischemic spinal cord injury and infraorbital injury in paper III and paper IV respectively. In paper III, we showed a significant sex difference in the development of acute allodynia-like behavior with female rats being more sensitive than male rats after ischemic spinal cord injury. This sex difference cannot be explained by the small and insignificant difference in mechanical sensitivity between the sexes before injury, nor by differences in the extent of injury. Our results agree with that of Gorman et al. (2001) who showed, using a rat model of spinal cord injury-induced spontaneous pain (the excessive grooming behavior), that although the characteristics of such behavior were similar between males and females, the female rats developed grooming with less spinal damage than males. Similarly, in paper IV, a significant sex difference was also found in the development of localized and wide spread mechanical hypersensitivity in rats after infraorbital nerve injury. These findings agree with our previous results using the same model (Dominguez et al, 2009).

One interesting observation from the work presented in Study III and IV is that the sex differences appear to be more prominent in wide-spread allodynia after spinal cord injury and infraorbital nerve injury (See also Dominguez 2009). As spinal cord injury-induced acute allodynia has been shown to be caused primarily by a dysfunction of the GABAergic inhibitory system in spinal cord (Hao et al, 1991 and 1992; Zhang et al, 1994), these results suggest that sex differences in the spinal GABAergic system may be an important underlying mechanism in sex differences in pain after spinal cord injury. Previous studies have shown that there is a close association between sex hormones and GABA in many aspects of neural function (Berkley, 1997). Estrogen increases GABA release, upregulates GABA receptors and increases the activity of glutamate acid decarboxylase (Kelly et al, 1992; Weiland, 1992; Saleh and Connell, 2003).

It is unclear what the mechanisms for sex difference after infraorbital injury. In the trigeminal region, there is an increased expression of glutamate NR2B subunit in masseter ganglion neurons in female rats compared with male rats after glutamate injection (Dong et al, 2007). Furthermore, greater afferent discharge has been found in females than males after glutamate injection into this area, which may be involve in the observed sex difference in the trigeminal region (Carins et al, 2002; Dong et al, 2007). Generally, the plasma level of sex hormones is not necessarily correlated with the level within the CNS levels (e.g. PAG, spinal cord and DRG) since sex steroids can be synthesized locally in the spinal cord (Murphy and Hoffman, 2001; Evrard and Balthazart, 2004; Evrard, 2006). It might be also the case for trigeminal complex due to the significant expression of steroidogenic enzyme (Horvath and Wikler, 1999). This might at least partly explain the absence of significant correlations between estrous phase and development of mechanical hypersensitivity. Clearly, further studies are needed to clarify the mechanisms in sex differences after spinal cord injury and infraorbital nerve injury.

5.5 ESTROUS STAGE AND THE DEVELOPMENT OF NEUROPATHIC PAIN-LIKE BEHAVIORS

The baseline sensitivity to mechanical stimuli of female rats was also tested at each estrous stage before nerve injury in papers III and IV. In paper III, the baseline nociception of the trunk area did not change across different estrous stages. Similarly, in paper IV the basal nociception in facial area and upper flank also did not exhibit

significant change related to different estrous stage. This suggested that different estrous stage has little effect on basal nociception under normal conditions in our testing regime.

Due to the fact that some painful conditions are related to menstrual cycle in humans (Greenspan et al, 2007), it is of interest to study whether there are estrous stage related pain in our experimental pain models. Studies with ovariectomized female rodents suggested a facilitatory role for female sex hormones in the development of pain-related behaviors following sciatic nerve and spinal cord injury (Colye et al, 1996; Gorman et al, 2001). From this perspective, we controlled the estrous stage at the time of injury and/or two time points after nerve injury to see if the development of hypersensitivity was affected by different estrous stages. In paper III, we observed that estrous stages at the time of spinal cord injury did not affect development of acute allodynia in female rats. In lines with this observation, different estrous stage at the time of infraorbital nerve irradiation was not found to influence the severity and duration of the hypersensitivity in either face or upper flank region in paper IV. This indicates that the magnitude of hypersensitivity is not dependent on specific estrous phase at the onset of the injury.

The changes of hypersensitivity across the estrous cycle were also examined in female rats in paper IV. The variation of hypersensitivity across the estrous cycle during a 15-day evaluation at 1-3 weeks and 12-14 weeks post nerve injury did not reveal an impact of estrous stage on mechanical sensitivity. However, the animals started to recover from the injury at 12-14 weeks at the face region, but not in the flank areas involved in spread sensitivity. Modulation of nociceptive behaviors in different stages of the estrous cycle has been reported previously in some, but not all, studies (Marks and Hobbs, 1972; Frey et al, 1993; Martinez-Gomez et al, 1994; Vincler et al, 2001). Vincler et al. (2001) showed that modulation of nociceptive behaviors during the estrous cycle is dependent on the type and duration of stimulus used. Thus it could be the same case in paper III and IV. Thus, the impact of estrous cycle on normal nociception and hypersensitivity to mechanical stimulus seems to be minimal in our testing paradigm.

6 CONCLUSIONS

1. ERα and ERβ are involved in determining sex difference in mechanical sensitivity under normal condition and after inflammation, but they do not appear to have a role in the development of mechanical hypersensitivity after sciatic nerve injury.

2. Activation of peripheral adenosine $A_{2A}R$ is involved in mechanical hyperalgesia after inflammation and the effect of $A_{2A}R$ activation is more profound in females than in males.

3. Females develop more profound acute mechanical allodynia after spinal cord injury which is independent of the extent of tissue injury or specific estrous phase at the time of injury.

4. There are sex differences in the development of localized and spread mechanical hypersensitivity after infraorbital injury in rats. However, there appears to be no change in hypersensitivity across estrous cycle in females.

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