

**SEX DIFFERENCES AND THE ROLE OF ESTROGEN, ESTROUS CYCLE,
AND DRG NEURONS IN A MOUSE MODEL OF CANCER PAIN**

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Chapter 1

Background and Research Overview

Cancer Prevalence and Pain

Cancer is second only to heart disease as a cause of mortality in the United States accounting for nearly 1 of every 4 deaths. According to the American Cancer Society over 1.5 million new cancer cases are expected to be diagnosed this year in the United States with over 500,000 patients projected to succumb to the disease [124]. With more than 10 million people diagnosed with cancer world-wide every year, it is projected that by 2020 this will have increased to 15 million new cases each year [16]. Cancer patients can experience a number of symptoms that include fatigue, lack of energy, weight loss, nausea, difficulty breathing, pain, anxiety, depression and problems sleeping [9]. Pain may be the first symptom that causes someone to seek medical advice that leads to a diagnosis of cancer. In cancer patients, pain may be caused by tumor progression and invasion, systemic treatments, cancer-related infections, and may involve inflammatory, neuropathic, ischemic, and compression mechanisms at multiple sites [68]. Although pain can occur at any time as the disease progresses, the frequency and intensity of pain tends to increase as the cancer advances. Compounding the complexity of treatment of patients with advanced

cancers is the development of bone metastases which occur in 65–80% of cancer patients with advanced cancer [28; 71]. Due to improved tumor control, patients are living longer with cancer thus increasing their susceptibility to bone metastases and as such, increasing the susceptibility to cancer pain that interferes with their daily living [50; 104]. A more thorough understanding of these mechanisms would certainly provide a foundation for the development of treatment regimens that can successfully relieve cancer pain in the majority of advanced cancer patients. However we are really in our infancy in terms of understanding the myriad molecular, cellular and systemic mechanisms that underlie the development and progression of cancer pain. The fact that bone cancer is so prevalent and represents one of the most painful types of cancer has lead us to focus on bone cancer in the studies summarized in this thesis.

Pathophysiology of bone metastasis

Bone cancer pain most commonly occurs when tumors originating in breast, prostate, or lung metastasize to long bones, spinal vertebrae, and/or pelvis. The development of bone metastases begins when primary tumor cells transverse the

walls of small blood vessels and enter circulation to transverse the wide-channelled sinusoids of the bone-marrow cavity. Tumor growth stimulates the activity of osteoclasts and osteoblasts, increases inflammatory markers and growth factors, reduces pH, and increases angiogenesis [28; 56; 116]. Tumor-induced bone remodeling enhances tumor growth and bone invasion, in turn creating devastating consequences on bone remodeling. These consequences include pathological bone fractures, pain, hypercalcaemia, and spinal cord and nerve-compression syndromes [65; 88; 89]. Bone metastases are usually associated with severe bone pain, which can be intractable [50]. Fluctuations in bone pain, called breakthrough pain, are transient spikes in pain often evoked by movement [105]. Since advances in cancer therapy are increasing life expectancy of the patient, the threat of severe pain can compromise the patient's quality of life. A reduction in pain levels can enhance mobility, in turn, strengthening bones to prevent further fractures, improve nutrition and enhance the quality of life and promote improved survival.

The guidelines of the World Health Organization's opioid analgesic ladder report cancer pain relief should be adequate in 75-90% of patients [138]. However, it has been reported that 45% of cancer patients have inadequate and undermanaged pain control [43; 46]. This inadequate pain treatment is due to various factors including incomplete implementation of the guidelines perhaps due to treatment-associated side effects. Opioids have adverse affects on various physiological functions including nausea, gastrointestinal motility, and suppressed respiratory activity [10]. Another major confound in treatment of cancer pain are discrepancies in analgesic treatment based on the sex of the cancer patient.

Sex differences in cancer pain

Sex plays a role in the vulnerability of humans to pain in experimentally-induced pain and in their sensitivity to analgesics [37; 46; 47; 49; 57]. The importance of sex differences in both acute and chronic pain conditions is demonstrated by the fact that women have a higher prevalence of several clinical and chronic pain conditions including fibromyalgia, arthritis, migraine, temporomandibular

disorders, and irritable bowel syndrome [48] [49]. Some debate still exists in the literature regarding sex differences in patients with cancer pain. Early reports failed to find sex differences in cancer pain [28; 137] but more recent studies reveal that sex differences do exist [18; 71]. Women are more likely to experience enhanced cancer pain due to reduced treatment with analgesics [93] leading to a lower quality of life compared to their male counterparts [56].

It is known that there are sex differences in cancer diagnosis with premenopausal women at greater risk to develop cancer compared to men of the same age [4]. This reverses after menopause with men being at greater risk to develop cancer compared to women. Male and female cancer rates therefore intersect around the age of the menopause and this phenomenon remains stable over time. The sex differences in cancer incidence could be attributed to the sex differences in hormonal status. Moreover, approximately 70% of cancer diagnoses are either lung, breast, or prostate cancer [16]. In the latter two cases, hormone status can exacerbate tumor growth due to the involvement of hormone-sensitive tissue [44; 51]. Overall, basic and clinical studies are just beginning to reveal the full extent

of sex differences in patients with cancer pain. A major focus of sex difference research is delineating the role of sex steroid hormones, specifically estrogens, involvement in cancer pain.

Estrogens and pain

When examining sex differences, effects of gonadal hormones are a major focus of research [8; 57]. Fluctuations in female hormones over the menstrual cycle correspond with changes in pain perception [39; 41; 49; 107; 112; 113]. Estrogens are considered a critical factor in sex-dependent differences in pain, where they have a complex role in inflammatory processes and pain responses [2; 49; 115; 118], reviewed in [25]. Estrogens exert effects by acting on two classic estrogen receptors, ER α and ER β , which are widely expressed throughout the spinal cord and dorsal root ganglion of both males and females [75; 99; 133; 139] where levels of expression fluctuate depending on endocrine status. Estrogens can act on ERs located in the plasma membrane or located in the nucleus [13]. Plasma membrane ERs also include a G protein coupled receptor (GPR30), which unlike ER α and ER β , does not change with hormonal

rhythms [85]. Membrane ER activation initiates a spectrum of intracellular signaling cascades [26; 90] and modulates neuronal membrane excitability by gating calcium currents producing a myriad of effects on nociception and antinociception [15; 26; 27; 35]. Estrogen receptors are located in dorsal root ganglion (DRG) neurons [99], thus suggesting estrogens acting at either the nucleus or on cell membranes can modulate a broad array of physiological functions in these cells including those involved in the pathophysiology of pain.

Estrogens have both pronociceptive and antinociceptive effects in a variety of animal models of pain. Estradiol is pronociceptive as it enhances capsaicin-induced acute pain in female rats [87] and potentiates sensitivity to colorectal distention [63]. Similarly, progesterone and estrogen receptor activation increases neuropathic pain in a mouse model of spinal nerve injury [72; 80]. Conversely, progesterone and estrogen receptor activation are antinociceptive in inflammatory models of pain in rats [63] [74]. Moreover, increases in estradiol concentration enhanced opioid antinociception in female rats [73; 127], and reversed thermal and mechanical hyperalgesia in ovariectomized rats [117] and

acted as an analgesic in a rat model of calculosis [1]. In cultures of DRG neurons, estradiol, acting on membrane receptors, attenuated ATP induced calcium currents [26] and eliminated the translocation and activation of PKC ϵ in response to β -adrenergic receptor activation [62]. These *in vitro* findings suggest that estradiol modulates DRG excitability. Whether estrogens and their receptors play a role in sex differences linked to cancer pain has yet to be determined.

Additional research is needed to clarify the underlying mechanisms of sex differences in pain to facilitate the development of new treatment modalities that improve cancer pain management for both men and women. The challenges facing new strategies to relieve cancer pain are multifaceted. One major limiting factor has been the lack of basic knowledge of the neurobiology of cancer pain. Until recently, interpretation of cancer pain modalities was limited to animal models of painful conditions other than cancer. Over the past decade, animal models of cancer pain have been developed and have begun to provide insight into the pathophysiology of bone cancer pain. These models are now available

to provide important information surrounding the mechanisms underlying sex differences in cancer pain.

Animal models of bone cancer pain

One of the first animal models of bone cancer pain involved the implantation of fibrosarcoma cells (NCTC 2472; derived from a spontaneous connective tissue tumor) into the medullary cavity of the femur or into the calcaneus bone of the hindpaw of C3H/He mice [19; 20; 140]. Implantation of NCTC 2472 tumor-inducing cells produces a bone cancer model with behavioral, cellular, and neurochemical changes that can be correlated with tumor growth and bone destruction within 21 days of cell implantation. Mice implanted with NCTC 2472 tumor cells exhibit nocifensive behavioral responses, including mechanical and thermal allodynia responses, that correlate with osteoclast bone destruction [19; 141]. With this animal model of bone cancer pain, initial studies show that tumor growth is associated with the release of inflammatory mediators that excite and sensitize nociceptors (e.g. substance P, NGF, osteoprotegerin ligand and interleukin-10), while tumor growth alters the morphology of peripheral nerve

fibers, leading to a shift in the nociceptive responses of primary afferent neurons [21; 122].

Primary afferent neuron perikarya are located in the DRG whose nerve fibers include unmyelinated C-fibers and thinly-myelinated A δ -fibers that arise from cell bodies that are 15–45 μ m in diameter. The single axon bifurcates into a peripheral branch that innervates peripheral target tissue and a central branch that enters the CNS to synapse on second order neurons. As tumor cells grow within and around the bone, the tumor impinges on surrounding tissue creating degradation and compression of peripheral nerve fibers thus causing nerve injury to the very distal processes of sensory fibers and leading to the development of neuropathic pain [58; 147]. Moreover, heightened excitability of peripheral nociceptors develops with the release of tumor-derived inflammatory products and the increase of tumor-induced acidosis [21; 120]. Morphological changes on the peripheral ends of nociceptors causes an increase in excitability called “peripheral sensitization.”

Peripheral sensitization is characterized by the increased activation of C and A δ nociceptors with mild noxious sensory stimuli being perceived as highly noxious, i.e. hyperalgesia, and normally non-noxious sensory stimuli being perceived as noxious stimuli, i.e. allodynia. Tumor-induced tissue injury produces retrograde signals to nociceptor neuron cell bodies in DRGs increasing the transcription of neuropeptides, growth factors, and signaling receptors such as transient receptor potential (TRP) channels in the nuclei of these injured sensory neurons. Taken together, these changes augment both central and peripheral sensitization, increasing spontaneous activity, and enhancing responsiveness to three modes of noxious stimulation: heat, cold, and mechanical stimuli

TRP receptors and cancer pain

TRP channels are predominantly expressed in C and A δ nociceptors and transmit noxious thermal, mechanical and chemical stimuli. TRP channels are modulated by pro-inflammatory mediators, neuropeptides and cytokines. As polymodal receptors that function at the peripheral nerve terminals and modulate synaptic transmission at the first sensory synapse between the DRG and dorsal

horn, these receptors are intrinsically involved in both peripheral and central sensitization of chronic pain states including bone cancer pain [34; 82; 108; 109]. Known to be expressed in primary afferent nociceptors and the focus of current drug development efforts (reviewed in [17]) , this thesis will focus on three major TRP receptors: TRPV1, TRPA1, and TRPM8.

TRPV1 - Molecular insights into the process of heat sensation came from the cloning and functional characterization of the vanilloid TRP (TRPV1) receptor that binds capsaicin, the main pungent ingredient in “hot” chili peppers [24]. TRPV1 receptor is a non-selective Ca^{2+} -permeable channel activated by capsaicin, vanilloid compounds, and multiple sensory stimuli including noxious heat (>42 °C), acid, and mechanical stimulation [91] [150] [142]. In male rodent models of bone cancer pain, TRPV1 receptor expression and activation significantly increase in DRG neurons [70; 97; 153] and correlate with nocifensive behaviors mentioned above. Conversely, in knock-out mice, *TRPV1*^{-/-}, these behaviors have been significantly attenuated leading researchers to consider TRPV1 receptor as a target for next generation analgesics [82].

Several clinically useful TRPV1 receptor antagonists and potent agonists have been synthesized and evaluated in animal models of cancer pain. Specifically, antagonists SB-366791 (GlaxoSmithKline) and JNJ 17203212 (Johnson and Johnson) and the potent agonists, high-dose capsaicin and resiniferatoxin (RTX, obtained from the cactus, *Euphorbia resinifera*), block or ablate TRPV1 receptor-expressing primary afferent fibers resulting in a loss of acute heat pain and mechanical sensitivity in animal models of bone cancer pain [95; 96; 108; 111; 134]. These results suggest that the TRPV1 channel has a role in the integration of nociceptive signaling in chronic pain states, like bone cancer, and that the modification of TRPV1 receptor might be effective in attenuating tumor-induced pain which is typically difficult to treat.

TRPA1 - TRP Ankyrin 1 (TRPA1) receptor is expressed in a subpopulation of nociceptors that also express TRPV1 receptors. TRPA1 receptor is activated by a diverse assortment of pungent or irritating reactive chemical compounds including those found in mustard oil (allyl isothiocyanate) and cinnamon oil

(cinnamaldehyde), as well as, cold and mechanical force stimuli [6; 33; 76-78; 129] eliciting a painful burning or prickling sensation. TRPA1 receptor has been shown to play a role in tumor growth [128]. In a rodent model of oral cancer, TRPA1 receptor expression increases in trigeminal nerves projecting to the tumor and it is suggested to play a role in tumor pain [149]. TRPA1 receptor's role in bone cancer pain is yet to be delineated. Research in animal models of other pain modalities, including inflammatory and neuropathic, show an important role of TRPA1 receptor in sensitization to both mechanical allodynia and cold hyperalgesia. Antagonists to TRPA1 receptor have been shown to reduce mechanical allodynia in rodent models of inflammatory pain [69; 81; 102]. Sex differences in TRPA1 receptor activation have been demonstrated with respect to cold sensitivity with females requiring TRPA1 receptor to elicit an initial noxious cold response, whereas following prolonged exposure to cold, both males and females will respond.

TRPM8 - TRP Melastatin 8 (TRPM8) receptor is expressed in a subset of both C and A δ nociceptors on both the peripheral and central terminals [109]. TRPM8

receptor is also expressed in human prostate cancer cells and may be used as a diagnostic marker or a target for cancer therapy [151; 152] , but it's role in bone cancer pain is still unknown. In animal models of inflammatory and neuropathic pain, TRPM8 receptor is involved in cold hypersensitivity [7; 31; 42; 131]. Menthol, the major agonist of TRPM8, is a well-known analgesic, present in many over-the-counter drugs including cough drops and topical pain creams. Menthol reduces nocifensive behaviors in rodents with intradermal capsaicin injection and rodents with spinal nerve ligation, suggesting a role for TRPM8 receptor activation to alleviate both acute and chronic pain conditions [108; 131].

TRP receptors are important sensors of mechanical, chemical, and thermal stimuli. During peripheral sensitization, DRG cell bodies receive nociceptor signals, which up-regulate the synthesis of TRP channels, thereby augmenting both central transmission and peripheral sensitization. Thus, nociception is influenced by changes in the number of TRP genes, their messenger RNAs, and protein products, along with their multimer organization. Using the accumulated research on inflammatory and neuropathic pain, one could speculate that the use

of TRPV1 receptor and TRPA1 receptor antagonists with TRPM8 receptor agonist could be useful to treat certain modalities of pain. Although clinical trials using TRPV1 receptor antagonists for cancer pain are underway, the roles of TRPA1 receptor and TRPM8 receptor in cancer pain are still unknown.

Overview of Thesis

While conflicting views exist in the literature regarding sex differences in cancer pain, recent studies show that women are more likely to experience greater cancer pain than men. Cycling sex steroid hormones are considered critical factors in sex-dependent differences in pain. Whether estrogens and their receptors play role in sex differences linked to cancer pain has yet to be determined. More specifically, a role for TRPV1 in bone cancer pain has been reported in males, but it is unknown what effect estradiol has on the *in vivo* function of TRPV1 in either sex. Moreover, the role of TRPA1 and TRPM8 receptors in bone cancer pain are unknown.

Using a well-established mouse model of bone cancer pain [141] we propose to:

1. Determine whether sex differences exist in tumor-induced nociception; 2.

Determine what effect estrogen has on this nociception; 3. Delineate the tumor-

induced expression of TRP mRNA in lumbar DRG cell bodies. Our overall

hypothesis is that estrogen potentiates cancer pain by altering tumor-induced

transcription of TRP receptors and their function. If estradiol and its effect on

TRP receptors contribute to sex differences in cancer pain, this will allow for

selective and specific treatment of male and female pain patients based not only

on sex, but also on hormonal status.

Chapter 2

Effects of Chronic Estrogen on Bone Cancer Pain in a Rodent Model: Sex
Differences, Estrogen Receptors and the Estrous Cycle

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Introduction

Sex plays a role in the vulnerability of humans to pain in experimentally-induced pain and in their sensitivity to analgesics [12; 14; 47; 55; 79; 94; 145]. The importance of sex differences in both acute and chronic pain conditions is demonstrated by the fact that women have a higher prevalence of several clinical and chronic pain conditions including fibromyalgia, arthritis, migraine, temporomandibular disorders, and irritable bowel syndrome [48; 49]. Some debate still exists in the literature regarding sex differences in patients with cancer pain. Early reports failed to find sex differences in cancer pain [28; 137] but more recent studies reveal that sex differences do exist [18; 71]. Women are more likely to experience enhanced cancer pain due to reduced treatment with analgesics [93] leading to a lower quality of life compared to their male counterparts [56]. Overall, basic and clinical studies are just beginning to delineate the full extent of sex differences in cancer pain.

When examining sex differences, effects of gonadal hormone are a major focus of research [8; 57]. Fluctuations in female hormones over the menstrual cycle

correspond with changes in pain perception [39; 41; 49; 107; 112; 113]. Estrogens are considered a critical factor in sex-dependent differences in pain, where they have a complex role in inflammatory processes and pain responses [2; 49; 115; 118], reviewed in [25]. The two classic estrogen receptors, ER α and ER β , are widely expressed throughout the spinal cord and dorsal root ganglion of both males and females, with specific fluctuations depending on endocrine status [75; 99; 133; 139]. In contrast, GPR30, a G protein-coupled intracellular transmembrane estrogen receptor, does not change with hormonal rhythms [85]. Progesterone and estrogen receptor activation increases neuropathic pain in a mouse model of spinal nerve injury [72], but they cause an antinociceptive effect in inflammatory and colorectal distention model of pain in rats [63; 74].

Whether estrogens and their receptors play role in sex differences linked to cancer pain has yet to be determined. In the present study, we utilized a mouse model of bone cancer [141] to delineate estrogen effects on cancer pain. We hypothesize that estradiol will potentiate tumor-induced mechanical hyperalgesia

and this effect is correlated with changes in estrogen or progesterone receptor expression.

Methods

Animals

Adult male and female C3H/He mice (8-10 wks of age) were obtained from the National Cancer Institute at NIH. Mice were housed under a 12-h light/dark photoperiod with food and water available ad libitum. All animal care was performed and supervised by the University of Minnesota Research Animal Resources. All procedures were reviewed and approved by the University of Minnesota Institutional Animal Care and Use Committee.

Surgery

To determine the effects of gonadal steroids, specifically 17β -estradiol (E), on

tumor nociception, a group of animals were gonadectomized (females: OVX, males: ORCH) with half receiving estrogen replacement (OVX+E, ORCH+E). On the day of surgery, animals were either gonadectomized, gonadectomized and given silastic-hormone implant, or subjected to sham surgery. Surgical procedures were carried out under aseptic conditions according to “Principles of Laboratory Animal Care” (NIH publication, 8th Ed, revised 2011). Mice were placed in an enclosed chamber and anesthetized with isoflurane. When the animal were not responsive to paw pinch, it was removed from the chamber and fitted with a facemask that continuously delivered 1–2% isoflurane in an air/oxygen mixture throughout the surgery. Ovariectomized females (OVX) had a 5-mm dorsal incision made through the skin and two lateral incisions made through the left and right abdominal muscle wall. Ligatures were placed around the oviduct and the ovary was removed above the ligation. The muscle wall was closed with silk sutures and the skin closed with staples. Orchestomized males (ORCH) had a 1-cm median incision through the scrotum and small incisions made through cremaster muscles to expose the testes. Ligatures were placed around the vas deferens and the testes were removed above the point of ligation.

Skin and muscles were closed with silk sutures. Sham surgery animals received identical surgical procedures, but without the ligation and removal of the gonadal organs.

For chronic estrogen replacement in OVX+E and ORCH+E animals, a 10-mm silastic implant (i.d.: 1.57mm, o.d.: 3.18mm; Dow Corning, MI) containing 3 mg of crystalline 17 β -estradiol (Sigma Chemical Co, St. Louis, MO) sealed at both ends with silicone medical adhesive was prepared and allowed to cure overnight. During gonadectomy surgery, the Silastic implant was placed subcutaneously (s.c.) through a small dorsal incision in the back. Previous studies have determined that such implants produce high physiologic levels of serum estrogen in circulation [30; 106].

Cell culture and implantation

Cell culture and tumor cell implantation were performed as previously described (Cain et al. (Cain, 2001 #27). Briefly, NCTC clone 2472 connective tissue cells were obtained from American Type Cell Culture. The cells were grown to

confluence in 75 cm² flasks in NCTC 135 medium, pH 7.35, 10% horse serum, and passed one time weekly by a 1:4–6 split ratio. The cells were then counted with a hemacytometer, pelleted, resuspended in PBS for implantation in a concentration of 2×10^5 cells/10 μ l. Mice were placed in an enclosed chamber and anesthetized with 1–2% isoflurane in an air/oxygen mixture. Cells were injected unilaterally into and around the calcaneus bone in a volume of 10 μ l, whereupon the syringe was used both to bore through the calcaneus bone (proximal to distal) and inject the cells as the needle was withdrawn. This implantation protocol was derived from a femur skeletal metastasis model that has been used to study the cellular and biochemical mechanisms mediating bone destruction at the tumor site [29].

Measurement of hyperalgesia

On the day of implantation, baseline values for mechanical and thermal sensitivity were determined for each animal randomly assigned to the cell-implanted or saline-injected groups, prior to hindpaw implantation of fibrosarcoma cells. Animals showing increased sensitivity to von Frey stimulation at baseline

were removed from this study. A 3.4 milliNewton (mN) von Frey monofilament was used because the responses of tumor-bearing C3H/He mice to this monofilament are reproducible and sufficiently large to allow detection of dose-dependent attenuation by analgesics or antagonists. Because naïve mice typically do not respond to this size filament [19; 141], this hyperalgesic response can also be classified as allodynia. Animals were placed on a wire mesh platform, covered with a hand-sized container, and allowed to acclimate to their surroundings for a minimum of 30 min before testing. The monofilament was applied 10 times (to the point of bending) on the plantar surface of each hindpaw. The number of vigorous responses to the monofilament were counted and expressed as a percentage of stimuli giving rise to a withdrawal response (percent response frequency). Following the von Frey test, ankle widths were obtained to determine tumor size. The tumor size was measured with a caliper and expressed in millimeters (mm). Tumor growth was determined by subtracting the baseline measurement from the final growth measurement on post implantation day 21. There were no sex differences in tumor growth between male and female C3H mice injected with NCTC 2472 fibrosarcoma cells

into the hind paw tumor nor were there any differences in tumor growth among gonadal-hormone groups when compared to intact mice (Table 1). Saline-injected control animals did not show any increases in ankle width nor in mechanical allodynia (data not shown).

Estrous cycle determination

The stage of the estrous cycle for individual female mice was determined using vaginal cytology. Samples were collected using swabs moistened with sterile saline; contents were smeared onto a slide and allowed to completely air-dry. Slides were placed in 95% EtOH for 5 min, and then stained in cresyl violet for 20-30 min. Slides were rinsed with water, allowed to air dry, and examined microscopically. Based on the cytologic profile, mice were deemed to be in one of two hormone states: proestrus/estrus (high hormone milieu with a predominance of nucleated epithelial cells or cornified squamous cells) or diestrus (low hormone milieu with a predominance of leukocytes) [8; 22].

Quantification of mRNA by real-time PCR

On post implantation day 21, dorsal root ganglia (DRG) L1–L5 were isolated from mice, placed in RNAlater (Qiagen) and stored at -80°C. Total RNA was isolated from DRG samples using RNeasy Lipid Tissue Mini Kits (Qiagen). RNA was reverse transcribed into cDNA using QuantiTect RT-PCR kits (Qiagen) as per the manufacturer's instructions. Real-time PCR experiments were performed with Perfecta SYBR Green Master Mix (Quanta) using Stratagene's Mx3000P Real-Time PCR Systems (Agilent Technologies). Each cDNA sample was run in triplicate for murine ER α , ER β , GPR30, and PGR and reference gene (β -actin). Primers were developed using NCBI Primer-BLAST (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) and synthesized at the BioMedical Genomics Center at the University of Minnesota. Primer pair sequences were as follows: ER α receptor (GenBank Accession number NM_001001445.1) forward primer 5'- TGATGAAAGGCGGCATACGG -3' and reverse primer 5'- TCAAGGACAAGGCAGGGCTA -3'; ER β (NM_177781.4) forward primer 5'- GCTGGGCCAAGAAAATCCCT -3' and reverse primer 5'- TGAGGACCTGTCCAGAACGA -3'; GPR30 (NM_134252.3) FORWARD PRIMER 5'- CTTTCATCATGCCCTTCGCCA- 3' and reverse primer 5'-

GCGAAGATCATCCTCAGGGC -3'; β -actin (NM_007393.3) forward primer 5'-
AGGAGTACGATGAGTCCGGC -3' and reverse primer 5'-
GCAGCTCAGTAACAGTCCGC -3'. PGR (NM_134252.3) FORWARD PRIMER
5'- CGTGTCGTCTGTAGTCTCGC- 3' and reverse primer 5'-
ACTGCTGGTCCCCTGTCTTT -3'; β -actin (NM_007393.3) forward primer 5'-
AGGAGTACGATGAGTCCGGC -3' and reverse primer 5'-
GCAGCTCAGTAACAGTCCGC -3'. The ratio of fold change in expression of the
mRNA of interest for each sample was calculated by normalization of cycle
threshold (Ct) values to β -actin. Using the equation derived by Pfaffl [103] to
correct for potential differences in PCR primer efficiencies between the target and
reference genes, Ct values for tumor-bearing animals were normalized to the
averaged Ct values calculated for saline-injected counterparts; Ct values for
saline-injected animals were normalized to the averaged Ct values calculated
from their own group. The fold change was then derived from the differences
between the Ct values ($2^{-(\text{del del Ct})}$).

Statistics

Results were analyzed with JMP software (v.9.0.0 Copyright © 2010 SAS Institute Inc., Cary, NC, USA). Values were represented as mean \pm SEM (standard error of the mean). Between group comparison of the mean frequency of withdrawal responses to mechanical stimuli were analyzed by one-way ANOVA with a Tukey-Kramer HSD. Between group comparison of the relative mRNA expression were analyzed by one-way ANOVA with a Tukey-Kramer HSD. Bivariate analysis was used to evaluate correlational data between relative mRNA expression and hyperalgesia responses. A value of $p < 0.05$ was considered statistically significant.

Results

In the present study, we used a well-characterized mouse model of bone cancer pain [141] to determine to what extent gonadal hormones contribute to tumor-induced nociception. At post implantation day (PID) 21, tumor-induced mechanical allodynia was assessed by the animals' responses to von Frey fibers (Fig 1). There were no significant differences between intact males and females ($79.3 \pm 3.3\%$ and $72.0 \pm 2.8\%$ respectively, $p=0.10$). However, females in the

proestrus/estrus phase of the estrus cycle had greater tumor-induced mechanical allodynia ($76.4 \pm 2.9\%$) than females in diestrus ($61.7 \pm 4.0\%$, $p=0.01$). To evaluate in more detail the effect of female sex hormones on tumor-induced nociception, we gonadectomized females (OVX) and replaced 17β -estradiol (OVX+E). Tumor-induced mechanical allodynia in OVX females ($67.5 \pm 7.5\%$) was similar to that observed in diestrus females ($p=0.98$). Conversely, OVX+E females ($90.0 \pm 4.1\%$) were more sensitive than intact females in the proestrus/estrus phase ($p=0.03$). These results indicate that 17β -estradiol contributes to enhanced mechanical allodynia in females.

Previous research has shown that circulating sex steroid hormones in males are associated with reduced nociception [54; 126], thus we examined the effect of gonadectomy on male tumor-induced nociception (ORCH) and the effect of 17β -estradiol replacement (ORCH+E). ORCH males had greater tumor-induced allodynia ($96.0 \pm 2.4\%$) than intact male counterparts ($p=0.005$). Conversely, ORCH+E males had reduced mechanical allodynia ($64.0\% \pm 5.1\%$, $p < 0.0001$) similar to OVX and diestrus females ($p=0.89$ and $p=0.72$, respectively). This

indicates an anti-nociceptive rather than a pro-nociceptive effect of estrogen in males.

To examine tumor-induced changes in estrogen and progesterone receptors, the fold change of receptor mRNA from lumbar DRG cell bodies was quantified at PID 21 (Fig 2). ER α mRNA expression differed significantly across experimental groups ($F_{(5, 47)}=4.52$, $p=0.00$) and a Tukey-Kramer HSD post hoc test showed that tumor-bearing OVX females had a greater relative fold expression of ER α (3.09 ± 0.48) compared to intact tumor-bearing males (-1.01 ± 0.37 , $p=0.01$) and ORCH males (-1.41 ± 1.02 , $p=0.01$, Fig 2A). There were no differences in ER β mRNA expression across experimental groups ($F_{(5, 47)}=173$, $p=0.15$, Fig 2B). However, there were differences in the relative expression of GPR30 ($F_{(5, 47)}=4.13$, $p=0.009$, Fig 2C) with tumor-bearing OVX females having a greater fold expression of GPR30 (3.49 ± 1.49) compared to tumor-bearing intact males (-1.11 ± 0.76 , $p=0.03$) and intact females (-1.32 ± 0.51 , $p=0.006$). When the phase of estrous cycle was factored into the analysis, proestrus/estrus females (-1.96 ± 0.41) have a greater fold decrease in GPR30 mRNA compared to OVX

(3.49±1.49), diestrus females (1.54±0.82), and ORCH males (1.71±0.26; p=0.0001, p=0.04, & p=0.02, respectively). PGR mRNA relative expression differed across groups ($F_{(5, 47)}=13.04$, p=0.00, Fig 2D) and was greatest in tumor-bearing females (8.21±2.43, p<0.0001) compared to all other groups. When the phase of estrous cycle was factored into the analysis of PGR mRNA expression, tumor-bearing proestrus/estrus females (10.15±2.68) had the highest fold expression compared to all other groups (p<0.0001). Whereas, diestrus females (5.19±0.04) had a higher relative expression compared to intact males (-2.54±0.96, p=0.001). These results suggest that estrogens effect the tumor-induced relative expression of estrogen and progesterone receptor mRNA.

To examine the full spectrum of von Frey fiber responses in treatment groups relative to their expression of mRNA for hormone receptors, we utilized statistical prediction to correlate tumor-induced mechanical allodynia and the tumor-induced changes in mRNA expression. Overall, ER α negatively correlated with tumor-induced mechanical allodynia (R=-0.331, p=0.023) with intact tumor-bearing females having the greatest negative correlation (R=-0.553, p=0.012, Fig

3A). Similarly, intact female GPR30 expression was negatively correlated with mechanical allodynia ($R=-0.566$, $p=0.0009$, Fig 3B). Conversely, there were no significant correlations for ER α or GPR30 and mechanical allodynia in intact males, gonadectomized, and gonadectomized with 17 β -estradiol replacement animals (data not shown). Moreover, ER β and PGR had no predictable effect (data not shown). Taken together, these relationships suggest a link between tumor-induced mechanical allodynia and the natural changes in sensitivity of DRG cells to estrogen.

Discussion

Based on evidence in the literature that estrogen increases nociception in animal models of pain [118], as previously reviewed [25], we hypothesized that tumor-induced bone cancer pain will increase in the presence of estrogen and, conversely, decrease when estrogen is removed. Furthermore, based on evidence that sex steroid hormones in males reduce nociception in models of pain [72], we hypothesized that both male and female nociception will vary based on the presence or absence of sex steroid hormones.

Our results provide several important and novel findings related to tumor-induced nociception and the effects of sex steroid hormones. First, we demonstrate that the stage of estrous cycle affects tumor-induced mechanical hyperalgesia with females in diestrus exhibiting reduced mechanical hyperalgesia compared to females in proestrus/estrus. To control cyclical effects of female sex steroid hormones, we removed the ovaries (OVX) and replaced 17β -estradiol (OVX+E). OVX animals have mechanical allodynia similar to diestrus cycling females. Mechanical allodynia is greater in OVX+E and proestrus/estrus females. These results reveal estrous cycle effects and indicate that female sex steroid hormones, specifically estradiol, enhance tumor-induced nociception.

In other models of pain, sex steroid hormones in males reduce nociception [54; 126]. Thus, we examined the effect of tumor-induced nociception on gonadectomized males (ORCH) and in males with 17β -estradiol replacement (ORCH+E). Consistent with previous findings, ORCH males have greater tumor-induced mechanical allodynia compared to either intact or estrogen replaced

counterparts. A novel finding in our work is that estrogen replacement (ORCH+E) reduced mechanical allodynia and was similar to females with reduced sex steroid hormones (diestrus/OVX). This suggests an opposite role for estrogen in males and females with tumor-induced nociception. Furthermore, these findings suggest that male's circulating hormones are necessary for reduced tumor-induced nociception and that estrogen plays a role in this phenomenon.

Previous research has shown that an increase in expression and activation of estrogen receptors will enhance nociception in rodent models of pain [35; 45; 64; 75; 83; 85; 125], while other research finds their expression and activation to be anti-nociceptive [23; 35; 86]. To delineate tumor-induced changes in estrogen and progesterone receptors, we examined ER α , ER β , GPR30, and PGR mRNA expression in lumbar DRG cell bodies. The removal of circulating hormones increased ER α and GPR30 expression in OVX females. While correlational analysis indicated intact female mechanical allodynia is negatively correlated with both ER α and GPR30 expression. Thus, the greater the ER α and GPR30

expression, the less sensitive tumor-bearing females are to von Frey stimulation. Agonists for ER α and GPR30 have been shown to reduce inflammatory pain in OVX and diestrus phase female rats [86]. Moreover, 17 β -estradiol can act directly on DRG cell bodies to reduce capsaicin induced TRPV1 receptor activation [148] and ER α knock-out mice are more sensitive to a formalin nociceptive response [35]. Together this work indicates an anti-nociceptive effect of ER α . Based on variations in estrogen receptor transcription over the estrous cycle, increases in mechanical nociceptive sensitivity during proestrus/estrus correspond with decreases in estrogen receptor synthesis. Conversely, decreased nociceptive sensitivity during diestrus corresponds with greater estrogen receptor expression. Thus, these relationships would suggest that estrogen treatment is antinociceptive during diestrus rather than during proestrus/estrus.

Progesterone increases neuropathic pain in male rodents [72], while other work has demonstrated that the combination of progesterone and estradiol produces an antinociceptive in female rodents [63; 74]. These contrasting findings could

be due to sex differences in the expression of PGRs during inflammatory and neuropathic pain states. Our work illustrates sex differences in tumor-induced PGR mRNA expression. Tumor-bearing females have an 8-fold increase in PGR mRNA, whereas, males have a -2.5 fold decrease. Progesterone supplementation reduces the antinociceptive effect of morphine in intact female rats [144]. Thus, enhanced mechanical allodynia and enhanced expression of the progesterone receptor during the proestrus/estrus of the estrous cycle would suggest that either a reduction in progesterone concentrations and/or supplementation a progesterone antagonist could be antinociceptive in tumor-bearing females.

Our findings in a mouse model of bone cancer indicate estrogens increase pain in females and reduce pain in males. Moreover, removal of estrogens effects tumor-induced changes of estrogen receptor mRNA in females, while increased female sex hormones increases progesterone receptor mRNA. Taken together, changes in both estrogen concentration and estrogen receptor effect tumor-induced nociception in females. In light of this, intact cycling females and the

examination of specific sex-steroid fluctuations is necessary to evaluate the development, maintenance, and possible analgesic paradigms on pain modalities including, but not limited to, cancer pain.

TABLE: ANKLE TUMOR SIZE

Group	Ankle Width (mm ± SEM)	Range	Sample size (n)
Males	4.11 ± 0.41	6.9 - 1.7	15
Females	4.22 ± 0.48	7.0 - 1.0	20
OVX	4.43 ± 0.83	6.8 - 2.9	4
OVX E	5.10 ± 0.39	5.9 - 4.3	4
ORCH	4.10 ± 0.94	6.7 - 2.0	5
ORCH E	3.74 ± 0.58	5.5 - 2.2	5

Table: Ankle tumor size

Summary of average tumor growth as measured on PID 21. The tumor size was measured with a caliper and expressed in millimeters (mm). Tumor growth was determined by subtracting the baseline measurement from the final growth measurement on post implantation day 21. Data is shown as group means ± SEM. Range of ankle width and sample size of all experimental groups are also shown.

FIGURE 1: Tumor-induced Mechanical Allodynia

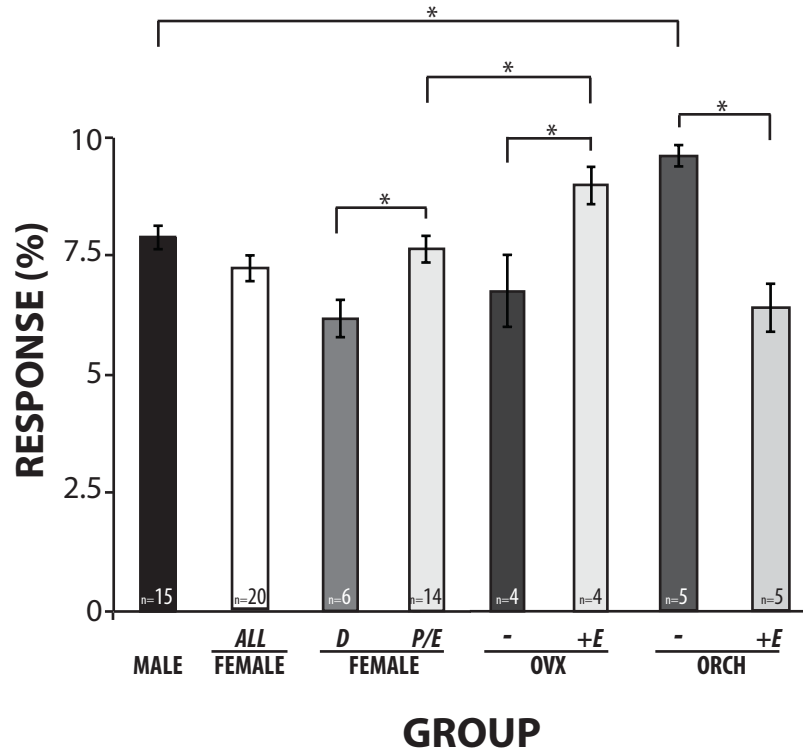


Figure 1: Von Frey mechanical allodynia
 Estrogen affects tumor-induced mechanical allodynia in both males and females. Von Frey fiber testing of the hind paw assessed mechanical allodynia. Intact males were statistically similar to intact females. Data are presented as group percent response means, % \pm SEM. *P<0.05

FIGURE 2: Tumor-induced mRNA expression

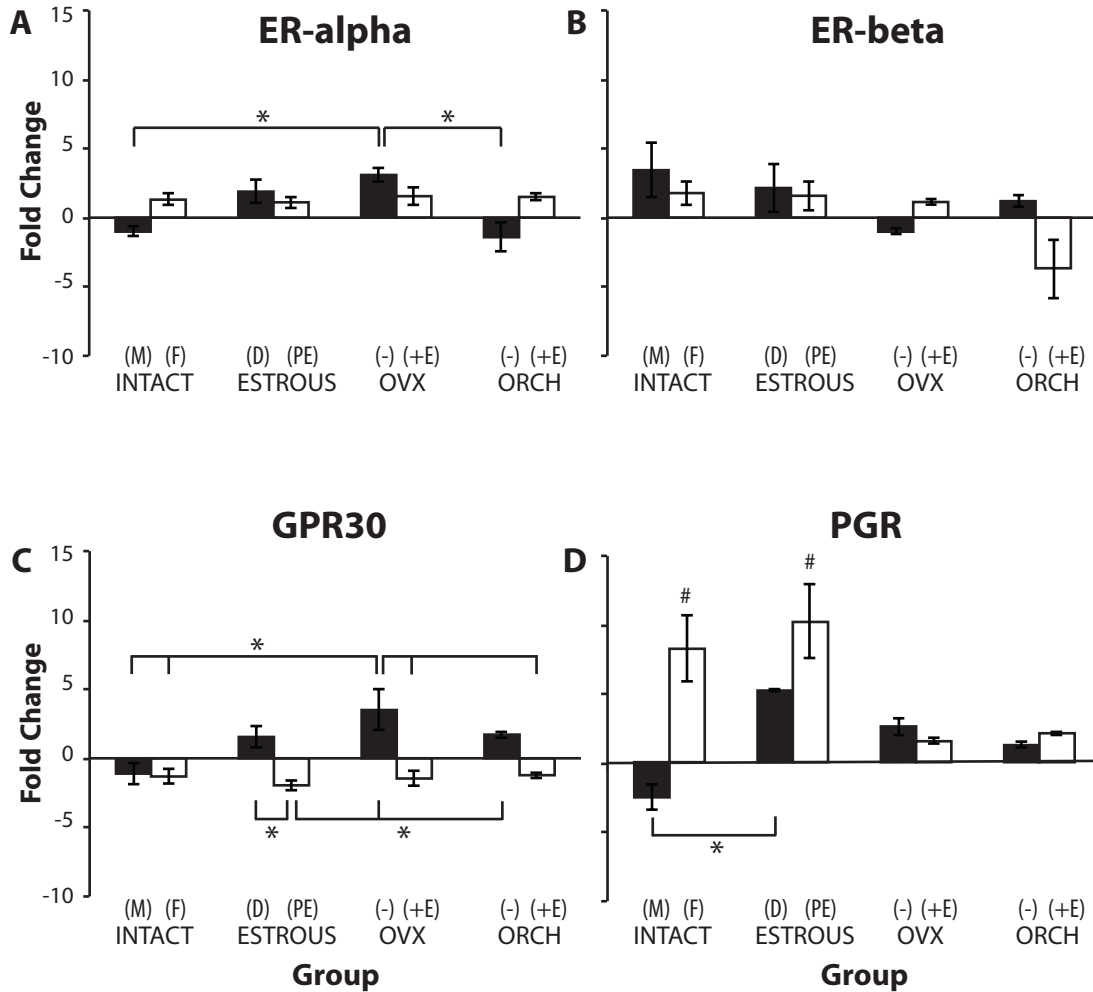


Figure 2: Relative mRNA expression

Sex and hormones status influence tumor-induced changes in lumbar dorsal root ganglia (DRG) ERalpha(A), GPR30 (C), and PGR (D), but no ERbeta(B)mRNA expression. Data are shown as mean fold change \pm SEM. * $P < 0.05$; # $P < 0.05$ compared to intact males, OVX, OVX+E, ORCH, and ORCH+E.

FIGURE 3: Correlational analysis of mechanical allodynia and relative mRNA expression

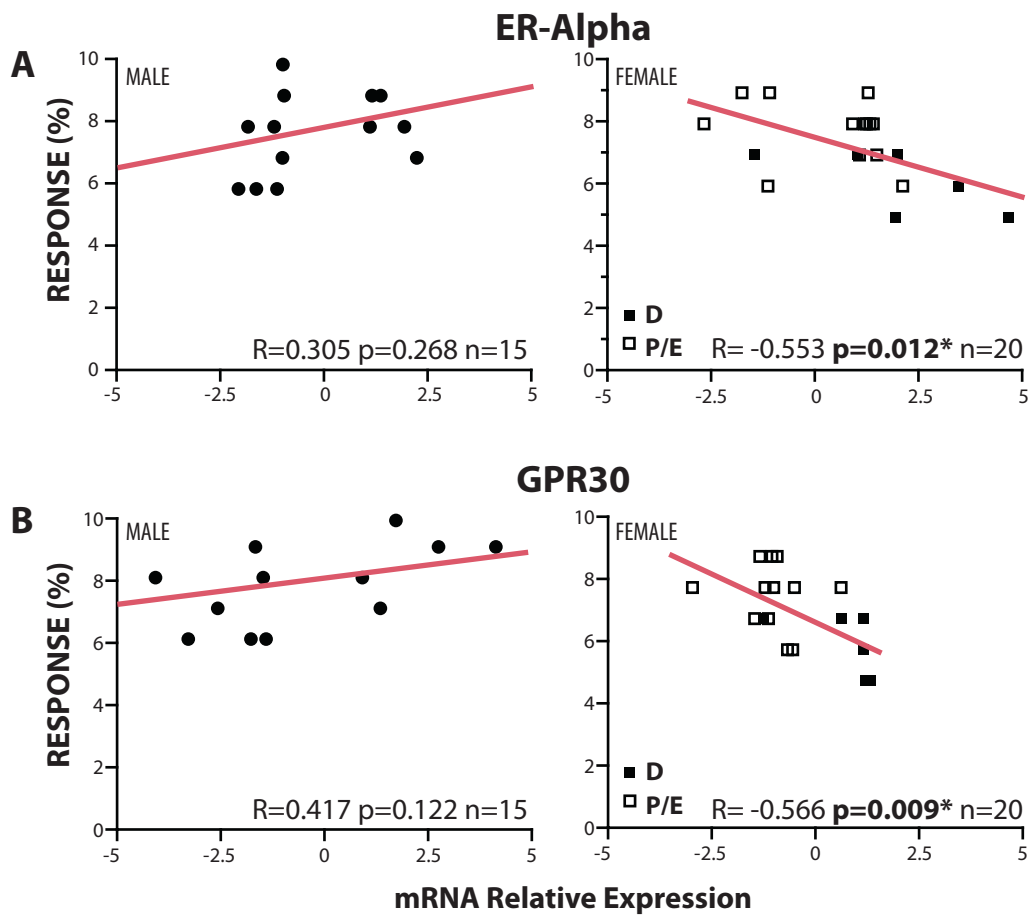


Figure 3:
 Correlational analysis of mechanical allodynia and relative mRNA expression
 Bivariate correlational analyses were used to determine the statistical significance of the relationship between tumor-induced mechanical allodynia and ERAlpha(A) and GPR30 (B) mRNA expression in intact males and intact females.
 * $P < 0.05$

Chapter 3

Estrogen effects on Bone Cancer Pain in a Rodent Model: Sex Differences,
TRPA1 and TRPM8, and Menthol-Induced Antinociception

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Introduction

Sex differences in the degree of pain experienced in a variety of human health conditions and some diseases are well documented in both clinical and experimental research [11; 47; 48; 57; 84]. Research is just beginning to delineate sex differences in cancer pain. Clinical reports indicate that women cancer patients have reduced quality of life due to reduced prescription of analgesics and, as a consequence, enhanced cancer pain [56; 93].

Malignant bone tumors occur not only in patients with primary bone cancer, but also as distant metastases from non-bone primary tumors, such as breast, prostate, and lung cancer. Tumor growth invades soft and boney tissue changing the chemical milieu, provoking inflammatory cell responses, and distorting a normally innocuous mechanical response. These changes in milieu sensitize nociceptors to both noxious and innocuous input [60; 61; 146]. During peripheral sensitization, dorsal root ganglion (DRG) cell bodies receive nociceptive signals which up-regulate the synthesis of transient receptor potential

(TRP) ion channels, thereby augmenting both central and peripheral sensitization (reviewed in [130])

An array of TRP ion channels are known to be involved with tumor-growth and cancer-induced nociception [108; 109]. Specifically, ankyrin-1 (TRPA1) is normally activated by a diverse assortment of irritating chemical compounds including those found in mustard oil (allyl isothiocyanate). TRPA1 is also activated by mechanical force stimuli [6; 76] as well as noxious cold [33; 67; 77; 129]. TRPA1 antagonists reduce pain responses in male rodents with tumor-tissue associated inflammation [135]. Relevant to the current study, TRPA1 expression increases in trigeminal nerves projecting to oral cancer in female mice [149].

Another TRP receptor, melastatin-8 (TRPM8) is involved in both cooling-mediated analgesia and cold hypersensitivity after injury in rodent models of pain [7; 31; 42; 131]. Menthol, a TRPM8 agonist, decreases nociception in both human patients and animal models of inflammatory and neuropathic pain [52; 66;

98; 136]. TRPM8 is expressed in human prostate cancer cells and may be used as a diagnostic marker or a target for cancer therapy [152].

Based on the role of TRP receptors in pain and the greater sensitivity to cancer pain in females than males, estrogen may influence TRP receptor activity.

Whether sex differences in cancer pain are due to the effects of sex hormones on TRPA1 and TRPM8 receptors has yet to be delineated. In the present study, we hypothesized that sex steroid hormones alter tumor-induced transcription of TRP receptors in DRG neurons as well as influence the response to TRPM8 sites to activation by menthol. Utilizing a mouse model of bone cancer [141], we compared intact, gonadectomized, and estradiol-replaced males and females to delineate gonadal hormones effect on tumor-induced TRPA1 and TRPM8 receptor expression and nociception. We also examined the antinociceptive effects of a topical application of menthol on tumor-induced mechanical allodynia and thermal hyperalgesia to determine if sex hormones, specifically estradiol, affects cooling-mediated analgesia.

Methods

Animals

Adult male and female C3H/He mice (8-10wks of age) were obtained from National Cancer Institute. Mice were housed under a 12 hour light/dark photoperiod with food and water available ad libitum. All animal care was performed and supervised by the University of Minnesota Research Animal Resources. All procedures were reviewed and approved by the University of Minnesota Institutional Animal Care and Use Committee.

Surgery

To determine the effects of gonadal steroids, specifically estradiol, on nociception, animals were gonadectomized (females: OVX, males: ORCH) with half receiving estrogen replacement (OVX+E, ORCH+E). Surgical procedures were carried out under aseptic conditions according to “Principles of Laboratory Animal Care” (NIH publication, 8th Ed, revised 2011). Mice were placed in an enclosed chamber and anesthetized with isoflurane in preparation for

gonadectomy. When the animals did not respond to paw pinch, it was removed from the chamber and fitted with a facemask that continuously delivered 1–2% isoflurane in an air/oxygen mixture throughout the surgery. On the day of surgery, animals were either gonadectomized, gonadectomized and given silastic-hormone implant, or subjected to sham surgery. Briefly, ovariectomized females (OVX) had a 5 mm dorsal incision made through the skin and two lateral incisions made through the left and right abdominal muscle wall. Ligatures were placed around the oviduct and the ovary was removed above the ligation. The muscle wall was closed with silk sutures and the skin closed with staples.

Orchectomized males (ORCH) had a 1 cm median incision through the scrotum and small incisions made through cremaster muscles to expose the testes. Ligatures were placed around the vas deferens and the testes were removed above the point of ligation. Skin and muscles were closed with silk sutures. Sham surgery animals received identical surgical procedures but without the ligation and removal of the gonadal organs.

For chronic estrogen replacement in OVX+E and ORCH+E animals, a Silastic-implant (i.d.: 1.57mm, o.d.: 3.18mm; Dow Corning, MI) containing 3 mg of crystalline 17 β -estradiol (Sigma Chemical Co, St. Louis, MO) sealed at both ends with silicone medical adhesive was prepared and allowed to cure over night. During gonadectomy surgery, the Silastic implant was placed subcutaneously (s.c.) through a small dorsal incision in the back. Previous studies have determined that such implants produce high physiologic levels of serum estrogen in the circulation [30; 106].

Cell culture and implantation

Cell culture and implantation were performed per Cain et al. [21]. Briefly, mice were placed in an enclosed chamber and anesthetized in preparation for cell implantation. When the animal did not respond to paw pinch, it was removed from the chamber and fitted with a facemask that continuously delivered 1–2% isoflurane in an air/oxygen mixture throughout the implantation. NCTC clone 2472 connective tissue cells were obtained from American Type Cell Culture.

The cells were grown to confluence in 75 cm² flasks in NCTC 135 medium, pH 7.35, 10% horse serum, and passed one time weekly by a 1:4–6 split ratio. The cells were then counted with a hemacytometer, pelleted, resuspended in PBS for implantation in a concentration of 2×10^5 cells/10 μ l. Cells were injected unilaterally into and around the calcaneus bone in a volume of 10 μ l, whereupon the syringe was used both to bore through the calcaneus bone (proximal to distal) and inject the cells as the needle was withdrawn. This implantation protocol was derived from a femur skeletal metastasis model that has been used to study the cellular and biochemical mechanisms mediating bone destruction at the tumor site [29].

Measurement of hyperalgesia

On the day of implantation, baseline values for mechanical and thermal sensitivity were determined for each animal randomly assigned to the cell-implanted or saline groups, prior to hindpaw implantation of fibrosarcoma cells. A 3.4 milliNewton (mN) von Frey monofilament was used because the responses of tumor-bearing C3H/He mice to this monofilament are reproducible and

sufficiently large to allow detection of dose-dependent attenuation by analgesics or antagonists [21; 141]. Testing was repeated throughout the time course of each study. Briefly, animals were placed on a wire mesh platform, covered with a hand-sized container, and allowed to acclimate to their surroundings for a minimum of 30 min before testing. The monofilament was applied 10 times to the point of bending on the plantar surface of each hindpaw. The number of vigorous responses to the monofilament were counted and expressed as a percentage of stimuli giving rise to a withdrawal response (percent response frequency).

Cold plate response latencies were obtained from mice placed on an aluminum plate cooled with ice ($1\pm 2^{\circ}\text{C}$). Latency to hind paw withdrawal/lift from the surface not related to general movements was determined as the end point. Mice were tested for a maximum of 30 s for each session. Data was recorded as threshold in s. There was at least a 30-min rest between mechanical and thermal testing. In a separate group of both fibrosarcoma-injected and naïve C3H mice, we applied menthol (10%, Biofreeze, Akron, OH) to the hindpaw plantar surface using cotton swab soaked with 500 μl of solution. Between 30-60

min following application, the latency of response to a von Frey fiber was obtained; 30 min after which, cold plate latencies were measured.

Following the cold plate test, ankle widths were obtained to determine tumor size.

The tumor size was measured with a caliper and expressed in millimeters (mm).

Tumor growth was determined by subtracting the baseline measurement from the measurement on that post implantation day. While tumor growth was evident, there were no sex differences in tumor growth between male and female C3H mice injected with NCTC 2472 fibrosarcoma cells into the hind paw tumor nor were there any differences in tumor growth among gonadal-hormone groups when compared to intact mice (data not shown). In saline-injected control animals the ankle width and mechanical nociceptive sensitivity did not change over the course of the experiments (data not shown).

Estrous cycle determination

Stage of the estrous cycle for individual female mice was determined using vaginal cytology. Samples were collected using swabs moistened with sterile

saline; contents were smeared onto a slide and allowed to completely air-dry. Slides were placed in 95% EtOH for 5 min, and then stained in cresyl violet for 20-30 min. Slides were rinsed with water, allowed to air dry, and examined microscopically. Based on the cytologic profile, mice were deemed to be in one of two hormones states: proestrus/estrus (high hormone milieu with a predominance of nucleated epithelial cells or cornified squamous cells) or diestrus (low hormone milieu with a predominance of leukocytes) [8; 22].

Quantification of mRNA by real-time PCR

Dorsal root ganglia L1–L5 were isolated from mice, placed in RNAlater (Qiagen, Valencia, CA) and stored at -80°C. Total RNA was isolated from DRG samples using RNeasy Lipid Tissue Mini Kits (Qiagen, Valencia, CA). RNA was reverse transcribed into cDNA using QuantiTect RT-PCR kits (Qiagen, Valencia, CA) as per the manufacturer's instructions. Real-time PCR studies were performed with Perfecta SYBR Green Master Mix (Quanta, Gaithersburg, MD) using Stratagene's Mx3000P Real-Time PCR Systems (Agilent Technologies, Santa Clara, California).

Each cDNA sample was run in triplicate for the murine TRPA1 and TRPM8 receptor and the reference gene (β -actin). Primers were developed using the NCBI Primer-BLAST (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) and synthesized at the BioMedical Genomics Center at the University of Minnesota. Primer pair sequences were as follows: TRPA1 (NM_177781.4) forward primer 5'- CCTCCCCGAGTGCATGAAAG -3' and reverse primer 5'- CCACATCCTGGGTAGGTGCT -3'; TRPM8 (NM_134252.3) FORWARD PRIMER 5'- TTTACCACGCACTGTGTCCC- 3' and reverse primer 5'- CGCATAGGAAATGGCGTTGC -3'; β -actin (NM_007393.3) forward primer 5'- AGGAGTACGATGAGTCCGGC -3' and reverse primer 5'- GCAGCTCAGTAACAGTCCGC -3'. The ratio of fold change in expression of the mRNA of interest for each sample was calculated by normalization of cycle threshold (Ct) values to β -actin. Using the equation derived by Pfaffl [103] to correct for potential differences in PCR primer efficiencies between the target and reference genes, Ct values for tumor-bearing animals were normalized to the averaged Ct values calculated for saline-injected counterparts; Ct values for

saline-injected animals were normalized to the averaged Ct values calculated from their own group. The fold change was then derived from the differences between the Ct values ($2^{(-\text{del del Ct})}$).

Statistics

Results were analyzed with JMP software (v.9.0.0 Copyright © 2010 SAS Institute Inc., Cary, NC, USA). Values were represented as mean \pm SEM (standard error of the mean). The mean frequency of withdrawal responses to mechanical stimuli and mean latency to respond for thermal stimuli were analyzed by repeated-measures ANOVA with Tukey-Kramer HSD or unpaired Student's t-test post hoc comparison. Between group comparison of the relative mRNA expression were analyzed by one-way ANOVA with a Tukey-Kramer HSD. A value of $p < 0.05$ was considered statistically significant.

Results

Mechanical allodynia was similar in both males and females ($F_{(1,28)}=0.6317$, $p=0.433$) as responses to the von Frey fiber increased similarly over time ($F_{(5,24)}=4.3436$, $p=0.0059$, Fig 1A). There was also a significant difference in tumor-induced mechanical allodynia between females in diestrus and females in proestrus/estrus ($F_{(1,13)}=9.9933$, $p=0.0075$, Fig 1B). These results suggest that circulating female sex steroid hormones associated with the estrous cycle enhance tumor-induced mechanical allodynia.

To examine estradiol's effect on tumor-induced nociception, we gonadectomized females (OVX) in one group of mice and replaced 17β -estradiol (OVX+E) in another. There was a pronociceptive effect of estradiol replacement ($F_{(1,6)}=6.1413$, $p=0.0479$) as tumor-induced mechanical allodynia was enhanced in OVX+E females compared to OVX females (Fig 1C). Because previous research has shown that circulating sex steroid hormones in males reduce nociception [54; 127], we examined the effect of gonadectomy (ORCH) and 17β -estradiol replacement (ORCH+E) on tumor-induced nociception in males. There was an antinociceptive effect of estradiol replacement on mechanical allodynia

($F_{(1,8)}=32.3025$, $p=0.0005$) as ORCH+E males had reduced mechanical allodynia (Fig. 1D). These results indicate that while there is no difference between intact males and females in sensitivity to mechanical pain, estrogen increases nociception in females but decreases it in males.

When examining tumor-induced thermal hyperalgesia in intact males and females, there was a significant effect of sex ($F_{(1,28)}=7.9259$, $p=0.0088$) that persisted over time ($F_{(5,24)}=2.8320$, $p=0.0379$). Females had a reduced threshold compared to males (Fig. 2A). There was no difference between tumor-induced thermal hyperalgesia in mice during proestrus/estrus compared to that during diestrus ($F_{(1,13)}=3.2325$, $p=0.0954$) except on PID 11 ($p=0.0465$, Fig 2B). The removal of circulating female sex hormones (OVX) and replacement of 17β -estradiol (OVX+E) had a significant effect over time ($F_{(5,30)}=3.3696$, $p=0.0156$, Fig 2C). Between groups, there was no effect ($F_{(1,6)}=1.3845$, $p=0.2839$), except at PID 21 when OVX+E females were more sensitive than OVX females ($p=0.0492$). The removal of circulating male sex hormones (ORCH) and replacement of 17β -estradiol (ORCH+E) each had a robust effect over time ($F_{(5,40)}=5.2056$, $p=0.0009$),

but there was no significant difference between these groups at any time tested ($F_{(1,8)}=0.2666$, $p=0.6196$, Fig 2D). Together, these results suggest females are more sensitive than males to tumor-induced thermal hyperalgesia and although male sex steroids had a protective effect in males, estrogen has little or no effect on thermal hyperalgesia in either sex.

Because TRPA1 expression increases in trigeminal nerves of mice with oral cancer [149], we examined the tumor-induced expression of TRPA1 in lumbar DRGs. TRPA1 mRNA expression differed significantly across experimental groups ($F_{(5, 47)}=14.6699$, $p<0.0001$) and post hoc tests showed that TRPA1 mRNA expression was less in tumor-bearing intact females than intact males (Fig 3A). There were no differences between diestrus and proestrus/estrus (data not shown). TRPM8 mRNA expression differed across experimental groups ($F_{(5,47)}=2.5501$, $p=0.0402$) such that intact males had less TRPM8 mRNA expression compared to intact females (Fig 3B). There were no differences between the phases of the estrous cycle (data not shown). These results indicate that there were sex differences in the tumor-induced expression of both

TRPA1 and TRPM8, but neither alteration of sex steroid hormones nor the replacement of 17β -estradiol had any effect on this expression.

To examine the anti-hyperalgesia effect of menthol in tumor-induced nociception, we applied 10% menthol to the plantar surface of the tumor foot. The change in von Frey responses (Fig 4A) and cold plate threshold (Fig 4B) are shown as group mean (\pm SEM). In all tumor-bearing animals, menthol reduced von Frey fiber responses. The hormone status of the animal is a factor in menthol-induced mechanical antinociception ($F_{(5,31)}=7.069$, $p=0.0002$) as OVX females and ORCH+E males had diminished antinociceptive effects compared to intact males, intact females, OVX+E, and ORCH (Fig 4A). In all tumor-bearing animals, menthol reduced von Frey fiber responses. The hormone status of the animal is a factor in menthol-induced thermal antinociception ($F_{(5,31)}=3.486$, $p=0.0129$) as OVX females had diminished antinociceptive effects compared to intact females, OVX+E, ORCH and ORCH+E (Fig 4A). The antinociceptive effect of menthol in OVX females did not differ from intact males. For both von Frey and cold plate assessments, there were no differences between the phases of the estrous

cycle on menthol antinociception (data not shown). Menthol had no effect in naïve, non-tumor-bearing, animals (data not shown). These results suggest that female sex steroid hormones are necessary for menthol to have an antinociceptive effect in tumor-bearing female mice.

Discussion

Our animal model of cancer pain increased von Frey fiber responses and cold plate latencies of response, indicating increased sensitivity to both tactile and thermal (cold) pain in both male and female mice. Females were more sensitive to the cold plate than males, but both were equally sensitive to the von Frey fiber. In spite of the lack of difference between males and females in their degree of mechanical allodynia, estrogen, either released endogenously during the estrus cycle or delivered after gonadectomy, increased von Frey fiber responses in females but decreased them in males. In contrast, cold-plate hyperalgesia was relatively insensitive to changes in estrogen, as indicated by the failure of the estrus cycle or addition of estrogen to gonadectomized mice to alter cold sensitivity. The greater sensitivity of females to cold is consistent with the fact

that females and female sex steroid hormones sensitize mice to cold [114; 119] and cold hyperalgesia [76].

Males and females also differed in their expression of cancer-induced changes in TRPA1 and TRPM8 expression, where intact males expressed more TRPA1 and intact females express more TRPM8. TRPA1 remained unchanged in males with hormonal manipulation while the decrease in TRPA1 in females was reversed by gonadectomy and not influenced further by estrogen. TRPA1 is considered a thermo-TRP that is gated by noxious cold and mechanical force [3; 76; 78]. TRPA1 has been implicated in mechanical allodynia in male rodent models of inflammatory pain [69; 102] [81] and cold plate and tail-flick experiments reveal TRPA1-dependent, cold-induced nociceptor behavior in mice [67]. The difference in TRPA1 mRNA expression between intact and gonadectomized females could be due to the influence of progesterone or other sex steroid hormones (e.g. leuteinizing hormone, follicle stimulating hormone). Moreover, we show that estradiol replacement does not affect the expression of TRPA1 suggesting other sex steroid hormones may influence TRPA1 mRNA expression.

Additionally, this phenomenon of less TRPA1 mRNA expression, but greater cold sensitivity in females compared to males could be due to a down-regulation of TRPA1 receptors in response to their enhanced activity. As mechanical allodynia and cold hypersensitivity increase, TRPA1 mRNA is decreased.

Tumor-induced TRPM8 mRNA expression was increased in females but was not influenced by hormonal manipulation. In males, the decrease in tumor-induced expression of TRPM8 was reversed following gonadectomy. Given that androgens affect the expression of TRPM8 mRNA in prostate tumor cells [5], androgens may similarly affect their expression in DRG neurons of tumor-bearing mice. The dissimilar changes of TRPM8 mRNA expression between intact males and females are consistent with the sensitivity of tumor-bearing mice to cold. Tumor-induced cold sensitivity differed by sex but was relatively insensitive to hormonal manipulation. This is comparable to rodent models of inflammation or nerve injury, where increased TRPM8-dependent hypersensitivity to cold develops together with increased TRPM8 expression and/or TRPM8

responsiveness in nociceptors [31; 110]. Taken together, this would suggest that testosterone attenuates cold sensitivity.

Menthol, a TRPM8 agonist, is a natural compound of plant origin that is anti-hyperalgesic at low concentrations [38; 66; 136; 143]. In our hands, activation of TRPM8 using menthol produced an antinociceptive effect, consistent with that indicated throughout the literature. To our knowledge, the present study is the first to show an antinociceptive effect of topical menthol on tumor-induced pain. The efficacy of menthol appears to be sex dependent. In females, the effects of menthol on cold and mechanical pain were decreased by OVX but restored by estrogen. In contrast, menthol-induced cold antinociception was not influenced greatly by gonadal hormones in males, yet mechanical antinociception was attenuated by addition of estrogen in ORCH males. Taken together, our results suggest menthol is a potential analgesic for tumor-induced nociception, but the antinociceptive effect depends on the hormonal status of the patient.

In conclusion, we identified sex differences in the tumor-induced expression of TRPA1 and TRPM8 in bone tumor-bearing mice and sex differences in cold sensitivity. The phase of the estrus cycle affects tumor-induced mechanical allodynia in intact females and estradiol replacement affects tumor-induced mechanical allodynia in an opposite manner between males and females. Menthol is antinociceptive in tumor-induced pain, an effect that depends on estrogen in females, but is attenuated by estrogen in males. With the development of TRPA1 antagonists to abate pain and the use of TRPM8 agonists and antagonists for cold pain therapies it is prudent to consider sex and hormonal status when evaluating analgesic effects on pain, including cancer pain.

Figure 1: Estrogen affects tumor-induced mechanical allodynia

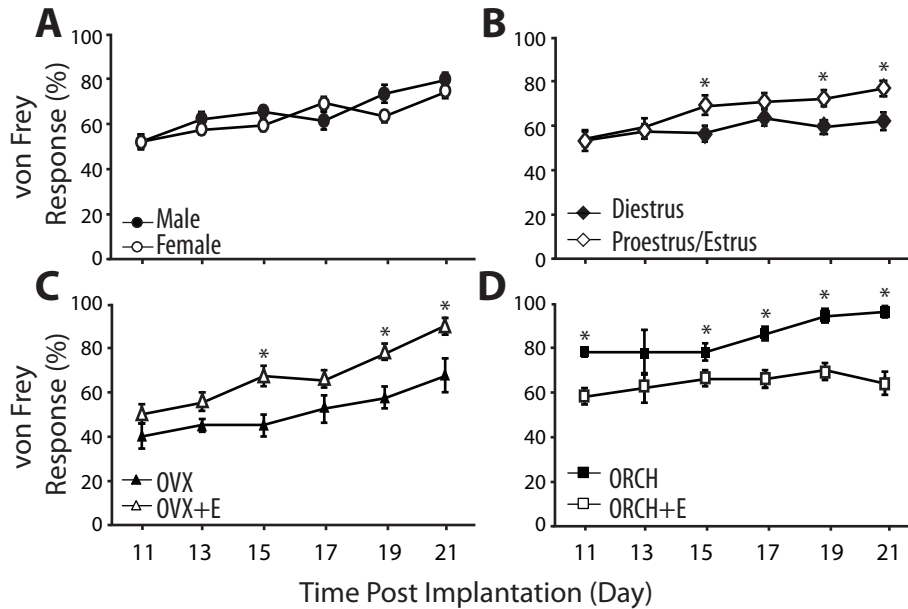


Figure 1: Estrogen affects tumor-induced mechanical allodynia in both males and females. Von Frey testing of the hind paw was used to assess tumor-induced mechanical allodynia (A) in males (n=15) and females (n=20) over time, (B) in females in the proestrus/estrus phase (n=9-14) compared to those in diestrus (n=6-11), (C) in OVX (n=4) and OVX+E females (n=4), (D) in ORCH males (n=5) and ORCH+E males (n=5). The group means (\pm SEM) of the data are expressed as percent of the response. Data were analyzed using repeated measures analysis of variance (ANOVA) followed by post hoc analysis using Tukey's HSD test where significance is indicated by an asterisk (P<0.05).

Figure 2: Females are more sensitive to cold nociception

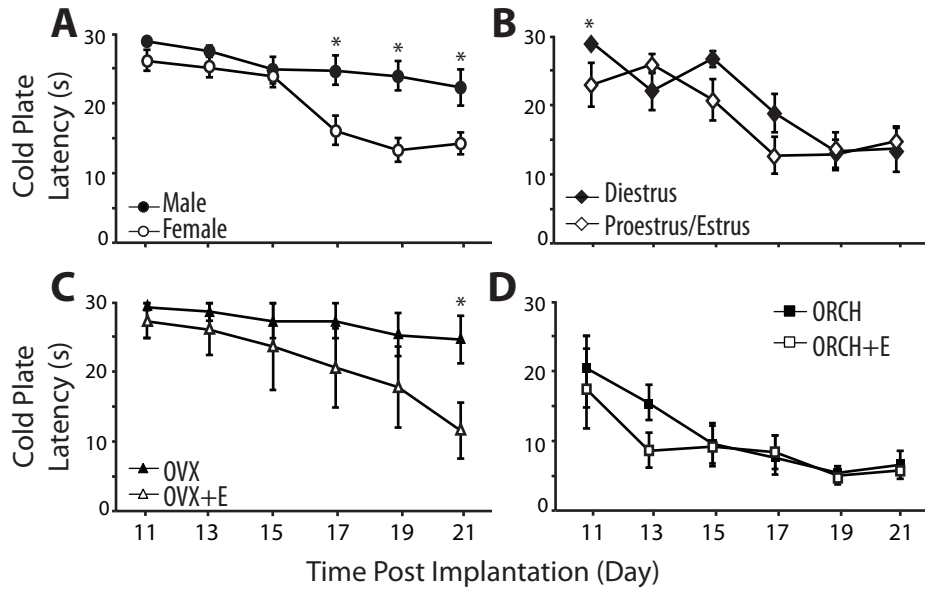


Figure 2: Cold nociceptive sensitivity is greater in females than males, but is unaffected by estrogen. Cold plate thresholds were used to assess the degree of thermal hyperalgesia (A) in females compared to males, (B) during the estrous cycle, (C) in OVX and OVX+E females, and (D) in ORCH and ORCH+E males. The group mean (\pm SEM) reflects the same n as in Figure 1. Data were analyzed statistically as in Figure 1.

Figure 3: Tumor-induced TRP mRNA expression

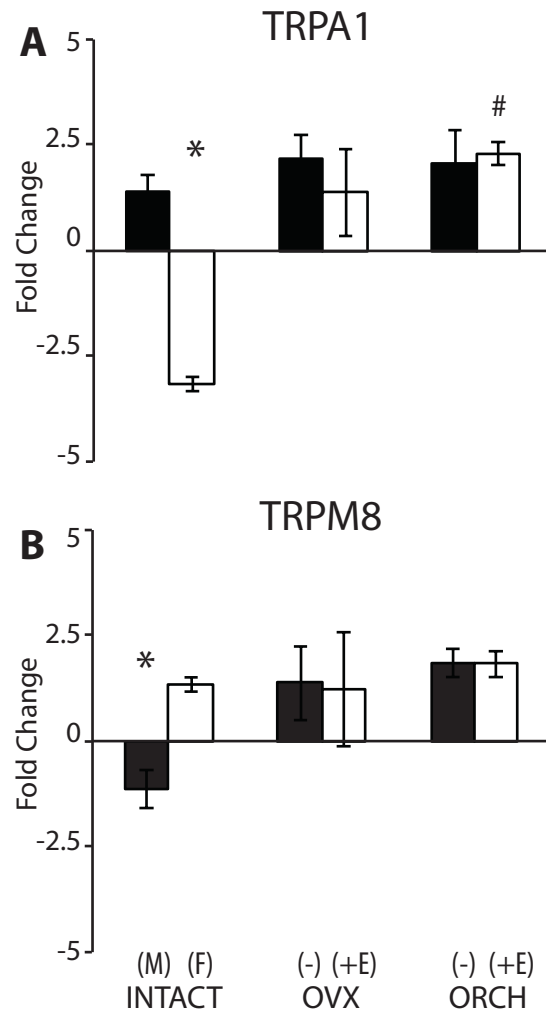


Figure 3: Tumor-induced TRP mRNA expression differs in males and females. Data indicate the relative expression of lumbar DRG TRPA1 (A) and TRPM8 (B) mRNA at PID 21. Lumbar DRG mRNA was assessed by comparing tumor-bearing animals to saline-injected animals of the same hormone status. The fold change is shown as group means (\pm SEM). Data were analyzed using a one-way ANOVA with Tukey HSD post hoc test where significance is indicated by an asterisk ($P < 0.05$). For sample numbers, refer to the legend for Figure 1.

Figure 4: Menthol reduces tumor-induced nociception

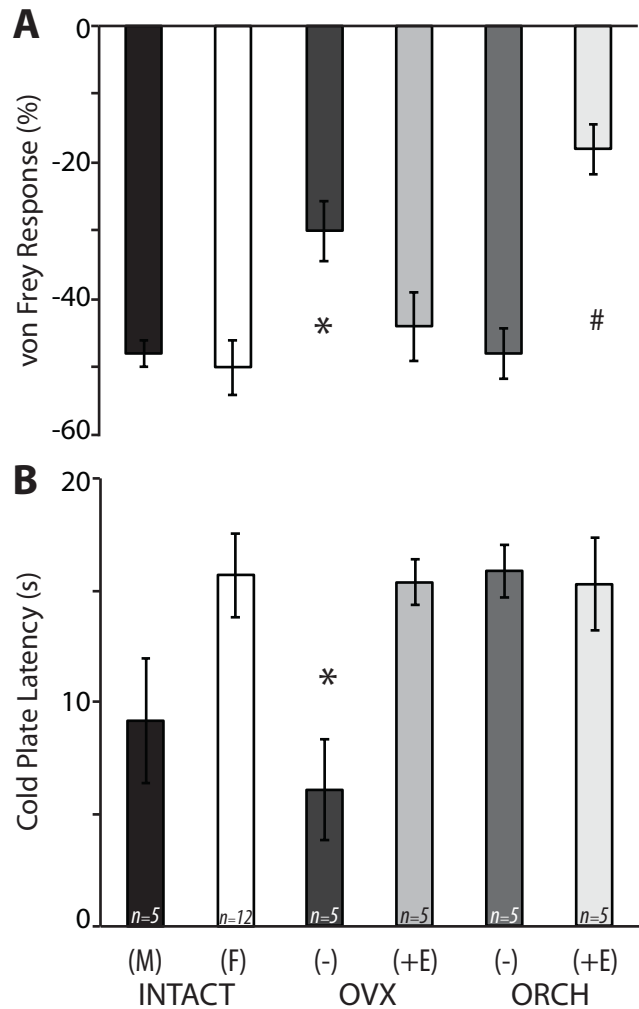


Figure 4: Menthol, a TRPM8 agonist, reduced tumor-induced nociception. The effect of plantar application of menthol on mechanical allodynia (A) and thermal hyperalgesia (B) was assessed in all groups. Antinociceptive responses were measured by subtracting pre-treatment nociceptive responses from the post-treatment assessments. Data are expressed as the mean (\pm SEM) changes in percent response (%) to a von Frey fiber and in the cold plate thresholds (sec). Data were analyzed statistically as in Figure 3.

Chapter 4

Sex differences and estrogen effects on TRPV1 expression and antagonism in a
mouse model of bone cancer pain

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Introduction

The study of sex differences has become of utmost importance for understanding and abating pain for numerous human health conditions and disease states [11; 47; 48; 57; 94]. Women self-report greater anxiety towards anticipated pain compared to their male counterparts [55] and have a higher prevalence of several chronic pain conditions and inflammation-mediated disorders including fibromyalgia, arthritis, migraine, temporomandibular disorders, and irritable bowel syndrome [48; 49]. Moreover, in experimentally-induced human pain testing, evidence exists for sex differences in sensitivity and in the response to analgesics [12; 14; 36; 47; 55; 79; 94; 145]. Basic and clinical studies are just beginning to delineate sex differences in cancer pain. In this regard, women are more likely to experience enhanced cancer pain due to reduced prescription of analgesics [93] and subsequently reduced quality of life compared to their male counterparts [56]. Thus, additional research is needed to clarify the mechanisms for sex differences to facilitate new treatment modalities that improve cancer pain management for both men and women.

Malignant bone tumors occur in patients with primary bone cancer and more commonly as distant metastases from non-bone primary tumors, such as breast, prostate, and lung cancer. As tumors grow they invade both soft and bony tissue changing the chemical milieu and provoking inflammatory cell responses, thus sensitizing nociceptors from being exclusively noxious stimulus detectors to detectors of innocuous inputs [60; 61; 146]. A number of cancer pain investigations have focused on members of the transient receptor potential (TRP) ion channel family [108; 109], since these receptors detect a diverse range of mechanical, chemical, and thermal stimuli and transduce both innocuous and noxious stimulus into action potentials in primary afferent nociceptors [34; 82; 100]. The first TRP channel discovered in mammalian sensory neurons was the vanilloid-1 (TRPV1) channel, which serves as the receptor for capsaicin, the pungent ingredient in hot chili peppers, and also acts as a key receptor for noxious physical heat (>42 °C) [24] and mechanical stimulation [91; 142; 150]. In male rodent models of bone cancer pain, increased nociceptive behavior is due to increased TRPV1 expression and activation in DRG neurons [70; 97; 153]. Conversely, in knock-out mice, TRPV1^{-/-}, these behaviors are significantly

attenuated (reviewed in [82]). Several clinically useful TRPV1 antagonists and potent agonists have been synthesized and evaluated in animal models of cancer pain (reviewed in [132]). These results suggest that the TRPV1 channel has a role in the integration of nociceptive signaling in chronic pain states, like bone cancer, and that the modification of TRPV1 might be effective in attenuating tumor-induced pain which is typically difficult to treat.

Whether sex differences in cancer pain are due to the effects of sex hormones on TRPV1 has yet to be delineated. Based on the role of TRPV1 receptors in pain and the greater sensitivity to cancer pain in females than males, estrogen may influence TRPV1 receptor expression and activity. In the present study, we hypothesized that sex steroid hormones alter tumor-induced transcription of TRPV1 channels in DRG neurons as well as influence the response of TRPV1 receptors to the TRPV1 selective antagonist, JNJ-17203212. Utilizing a mouse model of bone cancer pain [141], we compared intact, gonadectomized, and estradiol-replaced males and females to delineate gonadal hormones effect on tumor-induced TRPV1 receptor expression and nociception. To determine if sex

hormones, specifically estradiol, affect receptor antagonist analgesia we examined the antinociceptive effects of a TRPV1 antagonist on tumor-induced mechanical allodynia and thermal hyperalgesia

Methods

Animals

Adult male and female C3H/He mice (8-10wks of age) were obtained from National Cancer Institute. Mice were housed under a 12-hour light/dark photoperiod with food and water available ad libitum. All animal care was performed and supervised by the University of Minnesota Research Animal Resources. All procedures were reviewed and approved by the University of Minnesota Institutional Animal Care and Use Committee.

Surgery

To determine the effects of gonadal steroids, specifically estradiol, on nociception, animals were gonadectomized (females: OVX, males: ORCH) with

half receiving estrogen replacement (OVX+E, ORCH+E). Surgical procedures were carried out under aseptic conditions according to “Principles of Laboratory Animal Care” (NIH publication, 8th Ed, revised 2011). Mice were placed in an enclosed chamber and anesthetized with isoflurane in preparation for gonadectomy. When the animal did not respond to paw pinch, it was removed from the chamber and fitted with a facemask that continuously delivered 1–2% isoflurane in an air/oxygen mixture throughout the surgery. On the day of surgery, animals were either gonadectomized, gonadectomized and given silastic-hormone implant, or subjected to sham surgery. Briefly, ovariectomized females (OVX) had a 5 mm dorsal incision made through the skin and two lateral incisions made through the left and right abdominal muscle wall. Ligatures were placed around the oviduct and the ovary was removed above the ligation. The muscle wall was closed with silk sutures and the skin closed with staples.

Orchectomized males (ORCH) had a 1 cm median incision through the scrotum and small incisions made through cremaster muscles to expose the testes. Ligatures were placed around the vas deferens and the testes were removed

above the point of ligation. Skin and muscles were closed with silk sutures. Sham surgery animals received identical surgical procedures but without the ligation and removal of the gonadal organs.

For chronic estrogen replacement in OVX+E and ORCH+E animals, a Silastic-implant (i.d.: 1.57mm, o.d.: 3.18mm; Dow Corning, MI) containing 3 mg of crystalline 17 β -estradiol (Sigma Chemical Co, St. Louis, MO) sealed at both ends with silicone medical adhesive was prepared and allowed to cure over night. During gonadectomy surgery, the Silastic implant was placed subcutaneously (s.c.) through a small dorsal incision in the back. Previous studies have determined that such implants produce high physiologic levels of serum estrogen in the circulation [30; 106].

Cell culture and implantation

Cell culture and implantation were performed per Cain et al. [21]. Briefly, mice were placed in an enclosed chamber and anesthetized in preparation for cell implantation. When the animal did not respond to paw pinch, it was removed

from the chamber and fitted with a facemask that continuously delivered 1–2% isoflurane in an air/oxygen mixture throughout the implantation. NCTC clone 2472 connective tissue cells were obtained from American Type Cell Culture. The cells were grown to confluence in 75 cm² flasks in NCTC 135 medium, pH 7.35, 10% horse serum, and passed one time weekly by a 1:4–6 split ratio. The cells were then counted with a hemacytometer, pelleted, resuspended in PBS for implantation in a concentration of 2×10^5 cells/10 μ l. Cells were injected unilaterally into and around the calcaneus bone in a volume of 10 μ l, whereupon the syringe was used both to bore through the calcaneus bone (proximal to distal) and inject the cells as the needle was withdrawn. This implantation protocol was derived from a femur skeletal metastasis model that has been used to study the cellular and biochemical mechanisms mediating bone destruction at the tumor site [29].

Measurement of hyperalgesia

On the day of implantation, baseline values for mechanical and thermal sensitivity were determined for each animal randomly assigned to the cell-

implanted or saline groups, prior to hindpaw implantation of fibrosarcoma cells. A 3.4 milliNewton (mN) von Frey monofilament was used because the responses of tumor-bearing C3H/He mice to this monofilament are reproducible and sufficiently large to allow detection of dose-dependent attenuation by analgesics or antagonists [21; 141]. Testing was repeated throughout the time course of each study. Briefly, animals were placed on a wire mesh platform, covered with a hand-sized container, and allowed to acclimate to their surroundings for a minimum of 30 min before testing. The monofilament was applied 10 times to the point of bending on the plantar surface of each hindpaw. The number of vigorous responses to the monofilament were counted and expressed as a percentage of stimuli giving rise to a withdrawal response (percent response frequency).

Hot plate response latencies were obtained from mice placed on a heated plate ($52\pm 2^{\circ}\text{C}$; Technilab Instruments, Inc., Pequannoch, NJ). Latency to hind paw withdrawal/lift from the surface not related to general movements was determined as the end point. Mice were tested for a maximum of 30 s for each session.

Data was recorded as threshold in s. There was at least a 30-min rest between mechanical and thermal testing.

Following the hot plate test, ankle widths were obtained to determine tumor size.

The tumor size was measured with a caliper and expressed in millimeters (mm).

Tumor growth was determined by subtracting the baseline measurement from the measurement on that post implantation day. While tumor growth was evident, there were no sex differences in tumor growth between male and female C3H mice injected with NCTC 2472 fibrosarcoma cells into the hind paw tumor (Figure 1A) nor were there any differences in tumor growth among gonadal-hormone groups when compared to intact mice (Figure 2A). In saline-injected control animals the ankle width and mechanical nociceptive sensitivity did not change over the course of the experiments (data not shown).

Treatment with the TRPV1 antagonist

In a separate group of both fibrosarcoma-injected and naïve C3H mice, we injected the mice with JNJ-17203212, a TRPV1 selective antagonist (30 mg/kg,

s.c., Tocris Bioscience, Bristol, UK), dissolved in 120 μ L of Kollipher HS-15 (BASF Corporation, Florham Park, NJ) and 680 μ L of 5% dextrose or an equivalent volume of vehicle alone. Ghilardi et al. have shown previously that the dose used in the current study causes no observable adverse effects in movement performance, general activity, and body weight [53]. Antagonist behavioral testing was done on PID 21 when there was significant tumor growth and hyperalgesia. Prior to antagonist or vehicle injection, animals were tested for von Frey response and hot-plate latency as described above (pre-treatment). A one-time injection of antagonist was administered s.c. and approximately 1h later von Frey response was measured; 30 min after which, hot-plate latency was measured (post-treatment). The difference between the von Frey and hot-plate latency pre-treatment and post-treatment were calculated. Administration of the vehicle in a separate group of tumor-bearing mice did not produce any adverse effects (data not shown). Administration of either the antagonist or the vehicle did not produce any effects in naïve animals (data not shown).

Estrous cycle determination

Stage of the estrous cycle for individual female mice was determined using vaginal cytology. Samples were collected using swabs moistened with sterile saline; contents were smeared onto a slide and allowed to completely air-dry. Slides were placed in 95% EtOH for 5 min, and then stained in cresyl violet for 20-30 min. Slides were rinsed with water, allowed to air dry, and examined microscopically. Based on the cytologic profile, mice were deemed to be in one of two hormone states: proestrus/estrus (high hormone milieu with a predominance of nucleated epithelial cells or cornified squamous cells) or diestrus (low hormone milieu with a predominance of leukocytes) [8; 22].

Quantification of mRNA by real-time PCR

Dorsal root ganglia L1–L5 were isolated from mice, placed in RNAlater (Qiagen, Valencia, CA) and stored at -80°C. Total RNA was isolated from DRG samples using RNeasy Lipid Tissue Mini Kits (Qiagen, Valencia, CA). RNA was reverse transcribed into cDNA using QuantiTect RT-PCR kits (Qiagen, Valencia, CA) as per the manufacturer's instructions. Real-time PCR studies were performed with Perfecta SYBR Green Master Mix (Quanta, Gaithersburg, MD) using

Stratagene's Mx3000P Real-Time PCR Systems (Agilent Technologies, Santa Clara, California).

Each cDNA sample was run in triplicate for the murine TRPV1 receptor and the reference gene (β -actin). Primers were developed using the NCBI Primer-BLAST (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) and synthesized at the BioMedical Genomics Center at the University of Minnesota. Primer pair sequences were as follows: TRPV1 receptor (GenBank Accession number NM_001001445.1) forward primer 5'- CCGAAGTGGAGCAGAAGAAG -3' and reverse primer 5'- GATCCTTGCGACATGGTCAAG -3'; β -actin (NM_007393.3) forward primer 5'- AGGAGTACGATGAGTCCGGC -3' and reverse primer 5'- GCAGCTCAGTAACAGTCCGC -3'. The ratio of fold change in expression of the mRNA of interest for each sample was calculated by normalization of cycle threshold (Ct) values to β -actin. Using the equation derived by Pfaffl [103] to correct for potential differences in PCR primer efficiencies between the target and reference genes, Ct values for tumor-bearing animals were normalized to the averaged Ct values calculated for saline-injected counterparts; Ct values for

saline-injected animals were normalized to the averaged Ct values calculated from their own group. The fold change was then derived from the differences between the Ct values ($2^{(-\Delta\Delta Ct)}$).

Statistics

Results were analyzed with JMP software (v.9.0.0 Copyright © 2010 SAS Institute Inc., Cary, NC, USA). Values were represented as mean \pm SEM (standard error of the mean). The mean frequency of withdrawal responses to mechanical stimuli and mean latency to respond for thermal stimuli were analyzed by repeated-measures ANOVA with Tukey-Kramer HSD post hoc comparison. Between group comparison of the mean frequency of withdrawal responses to mechanical stimuli and mean latency to respond for thermal stimuli were analyzed by one-way ANOVA with a Tukey-Kramer HSD or an unpaired Student's t-test post hoc comparison. Between group comparison of the relative mRNA expression were analyzed by one-way ANOVA with a Tukey-Kramer HSD. Bivariate analysis was used to evaluate correlational data between relative

mRNA expression and hyperalgesia responses. A value of $p < 0.05$ was considered statistically significant.

Results

Mechanical allodynia was similar in both males and females ($F_{(1,28)}=0.0338$, $p=0.339$) as responses to the von Frey fiber increased similarly over time ($F_{(9,20)}=39.48$, $p<0.0001$, Fig 1B). There was a greater tumor-induced mechanical allodynia in females during the proestrus/estrus phases of the estrous cycle than during diestrus ($F_{(1,13)}=0.369$, $p=0.047$, Fig 1C). These differences started at PID 15 and were most robust at PID 21. Hot plate latencies were similar in both males and females ($F_{(1,18)}=0.015$, $p=0.607$, Fig 1D) with no detectable change over time ($F_{(9,10)}=1.74$, $p=0.20$). The phase of estrous also did not affect thermal hyperalgesia ($F_{(1,6)}=0.012$, $p=0.917$, Fig 1E). These results indicate that while tumor-induced thermal hyperalgesia does not develop, estrogen enhances tumor-induced mechanical allodynia.

Given the robust difference in mechanical allodynia at PID 21, we focused our remaining studies on this time-interval. To examine estradiol's effect on tumor-induced nociception, we gonadectomized one group of females (OVX) and replaced 17β -estradiol (OVX+E) in half of these mice. Estradiol replacement (OVX+E) increased tumor-induced mechanical allodynia compared to OVX alone ($p=0.033$, Fig 2B). Because sex steroid hormones reduce nociception in males [54; 127], we also examined the effect of gonadectomy (ORCH) and 17β -estradiol replacement (ORCH+E) on tumor-induced nociception in males. There estradiol replacement (ORCH+E) reduced mechanical allodynia compared to ORCH alone ($p=0.0005$, Fig. 2B). Neither tumor growth (Fig 2A) nor hot plate latency (Fig 2C) was influenced by gonadectomy and estradiol replacement in either males or females (Fig 2C). These results indicate that while there is no difference between intact males and females in sensitivity to mechanical pain, estrogen increases mechanical nociception in females, but decreases it in males.

TRPV1 expression increases in DRG neurons of male mice [70] and male rats [121] with bone cancer. However, it is not known whether hormone status of the

tumor-bearing animal effects TRPV1 expression. We found TRPV1 mRNA expression was greater in tumor-bearing intact females during proestrus/estrus than during diestrus; (Fig 3A, $F(5, 47)=3.035$, $p=0.0187$). This suggests that increases in circulating estrogen increase tumor-induced TRPV1 mRNA expression. Conversely, OVX females have greater TRPV1 expression than OVX+E females and ORCH+E males, suggesting that replacement of estradiol decreases tumor-induced TRPV1 expression. These results indicate that the availability of estrogen can affect tumor-induced expression of TRPV1 in an opposite manner.

Using correlational analyses, we found that in intact tumor-bearing females, TRPV1 positively correlated with tumor-induced mechanical allodynia ($R=0.602$, $p=0.005$, Fig 3B). Conversely, there was no correlation between these parameters in intact tumor-bearing males ($R=0.047$, $p=0.868$). There were no similar correlations between TRPV1 and mechanical allodynia in gonadectomized and gonadectomized with 17β -estradiol replacement animals (data not shown). Moreover, there was no correlation between TRPV1 and

thermal hyperalgesia (data not shown). Taken together, these relationships suggest a link between tumor-induced mechanical allodynia and changes in mechanical sensitivity of DRG cells to estrogen in intact females.

In all tumor-bearing animals, acute treatment with 30 mg/kg s.c. of JNJ-17203212, a TRPV1 receptor antagonist, reduced von Frey fiber responses (Fig 5A). The hormonal status of the mice is a factor in JNJ-17203212-induced mechanical antinociception ($F(5,30)=8.303$, $p=0.0003$) as females in the proestrus/estrus phase of the estrous cycle have greater mechanical antinociception than females in the diestrus phase, and greater than intact males, OVX, OVX+E, and ORCH+E mice. Conversely, ORCH+E males had diminished antinociception in response to JNJ-17203212 compared to intact females, OVX+E, and ORCH mice (Fig 5A). TRPV1 receptor antagonism induced thermal analgesia with no influence by hormonal status or sex of the experimental group (Fig 5B). JNJ-17203212 had no effect in naïve or non-tumor-bearing animals (data not shown). These results suggest that estrogen is

necessary for JNJ-17203212 to have an antinociceptive effect in tumor-bearing female mice, but estrogen can inhibit this antinociceptive effect in males.

Discussion

In contrast to inflammatory models of pain showing that females are also more sensitive to mechanical hyperalgesia than males [32; 40], there are no sex differences in the intensity of mechanical hyperalgesia in an osteosarcoma model of bone cancer pain [123]. Consistent with this, in our animal model of fibrosarcoma bone cancer pain, males and females have the same sensitivity to mechanical and thermal (heat) nociception. However, estrogen, either released endogenously during the estrus cycle or delivered after gonadectomy, increased von Frey fiber responses in females but decreased them in males. Hot-plate hyperalgesia did not develop in tumor-bearing animals, consistent with previous findings indicating that tumor-bearing mice do not develop thermal hyperalgesia until 4 weeks (28 days) post implantation [92], well beyond the PID 21 that we examined.

In models of bone cancer pain, TRPV1 expression and activation is increased in DRG neurons of males [59; 70; 97; 135; 153]. In our model of fibrosarcoma bone cancer pain, we are the first to demonstrate that females also have an increase in TRPV1 mRNA expression in DRG neurons innervating the hindpaw similar to their male counterparts. During the estrous cycle, nociception in other models of pain [37; 63] and TRPV1 sensitivity [101] both vary. We also found that the estrous cycle influences fibrosarcoma bone cancer pain as TRPV1 receptor mRNA expression differed between the two estrous cycle groups. Females in diestrus have lower TRPV1 mRNA expression whereas those in proestrus/estrus express nearly a 5-fold increase in TRPV1 mRNA expression. Conversely, gonadectomy increased TRPV1 mRNA in females, while estrogen replacement reversed this. The difference in TRPV1 mRNA expression between intact and gonadectomized females could be due to the influence of progesterone or other sex steroid hormones (e.g. leuteinizing hormone, follicle stimulating hormone). We show that estradiol replacement does not affect the expression of TRPV1 in males and females, adding to the suggestion that other sex steroid hormones influence TRPV1 mRNA expression. This phenomenon of less TRPV1 mRNA

expression, but greater mechanical sensitivity in OVX+E females compared to OVX females could be due to a down-regulation of TRPV1 receptors as a result of their enhanced activity. As mechanical allodynia increased, TRPV1 mRNA decreased, yet correlational analysis indicated intact female mechanical allodynia is positively correlated with TRPV1 expression.

In models of bone cancer pain, TRPV1 receptor antagonists reduce cancer pain in male rodents [53; 95-97; 121; 135]. Specifically, JNJ-17203212, attenuates both ongoing and movement-evoked nocifensive behaviors in male mice with bone cancer pain [53]. In our model of fibrosarcoma bone cancer pain, antagonism of the TRPV1 receptor also attenuated mechanical allodynia in females but had no effect on thermal hyperalgesia in both sexes. The efficacy of JNJ-17203212 appeared to be estrogen-dependent as JNJ-17203212 reduced mechanical pain during proestrus and estrus but attenuated mechanical antinociception by the addition of estrogen in ORCH males. It is known that 17β -estradiol can act directly on DRG cell bodies to reduce capsaicin-induced TRPV1 receptor activation [148]. Taken together, our results suggest that JNJ-

17203212 is a potential analgesic for tumor-induced nociception, but the antinociceptive effect depends on the recipients' hormonal status.

In conclusion, estrogen affects tumor-induced expression of TRPV1 and the phase of the estrus cycle determines the magnitude of tumor-induced mechanical allodynia in intact females. Estradiol replacement affects tumor-induced mechanical allodynia in an opposite manner in males than in females. JNJ-17203212 is antinociceptive on tumor-induced pain, an effect that depends on estrogen in females, but is attenuated by estrogen in males. Future use of TRPV1 antagonists to abate bone cancer pain must take into account sex and hormonal status.

Figure 1: Estrous cycle affects mechanical allodynia but not thermal hyperalgesia.

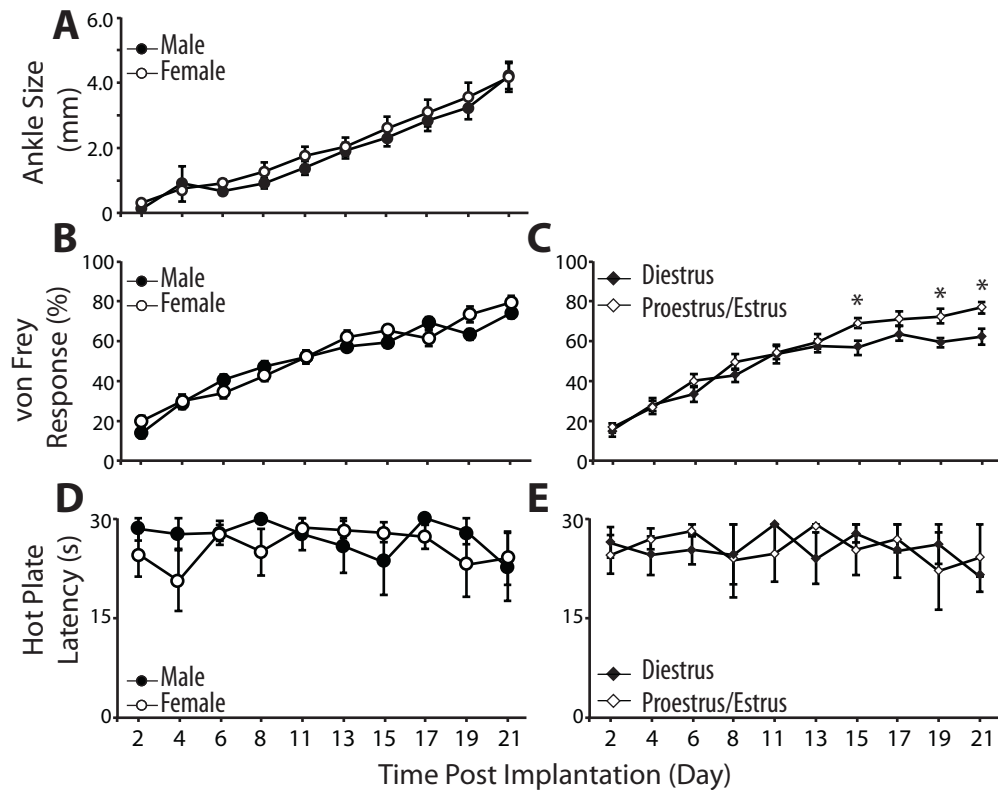


Figure 1: Tumor growth was measured by ankle width (A) in males (n=15) and females (n=20) over time. Von Frey testing of the hind paw was used to assess tumor-induced mechanical allodynia (B) in males and females over time, (C) in females in the proestrus/estrus phase (n=9-14) compared to those in diestrus (n=6-11). The group means (\pm SEM) of the data are expressed as percent of the response. Hot plate latency were used to assess the degree of thermal hyperalgesia (D) in females compared to males and (E) during estrous cycle. The group means (\pm SEM) of the data are expressed as latency in seconds. Data were analyzed using repeated measures analysis of variance (ANOVA) followed by post hoc analysis using Tukey's HSD test where significance is indicated by an asterisk ($P < 0.05$).

Figure 2: 17beta-estradiol replacement effects nociception in gonadectomized mice.

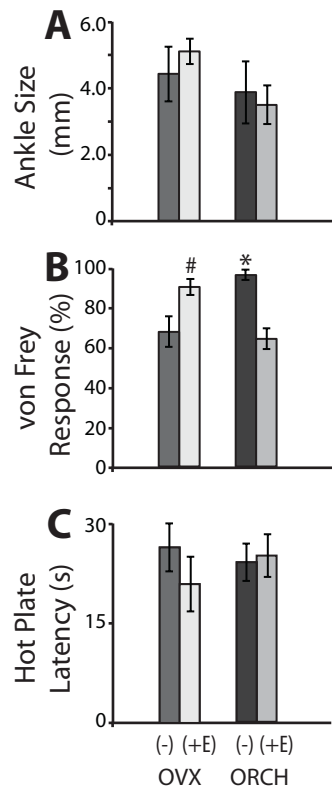


Figure 2: Tumor growth was measured by ankle width (A) in OVX (n=4), OVX+E (n=4), ORCH (n=5), ORCH+E (n=5) at post implantation day (PID) 21. Von Frey testing of the hind paw was used to assess tumor-induced mechanical allodynia (B) and hot plate latency was used to assess the degree of thermal hyperalgesia (C) in OVX, OVX+E, ORCH, ORCH+E at PID 21. The group means (\pm SEM) of the data are shown. Data were analyzed using a One-Way ANOVA followed by post hoc analysis using Tukey's HSD test. Hash tag (#) indicates OVX+E is significant compared to ORCH+E and OVX where significance indicated by an asterisk (*) indicates ORCH is significant compared to ORCH+E and OVX ($P < 0.05$).

Figure 3: Estrous cycle and estrogen affects the tumor-induced TRPV1 mRNA expression in females.

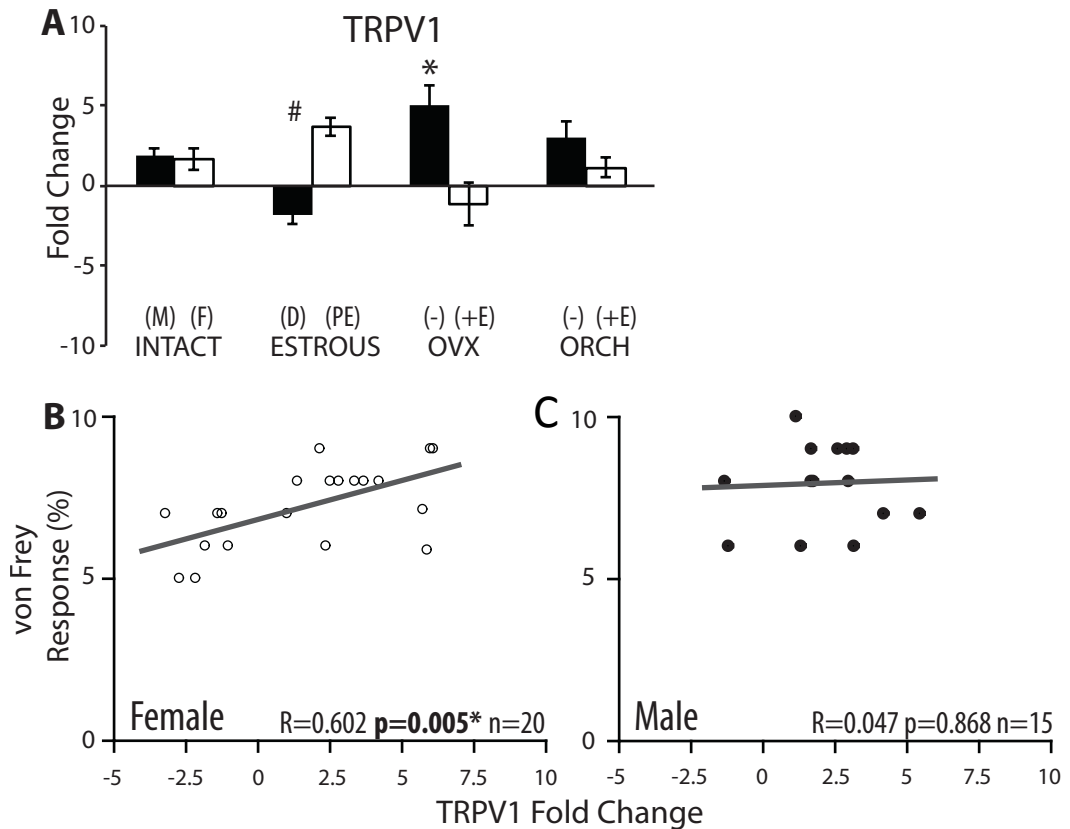


Figure 3: Data indicate the relative expression of lumbar DRG TRPV1 (A) mRNA at PID 21. Lumbar DRG mRNA was assessed by comparing tumor-bearing animals to saline-injected animals of the same hormone status. The fold change is shown as group means (\pm SEM). Data were analyzed using a one-way ANOVA with Tukey HSD post hoc test. Hash tag (#) indicates females in the diestrus phase of the estrous cycle are significant compared to proestrus/estrus females, intact males, OVX, and ORCH, where significance indicated by an asterisk (*) indicates OVX is significant compared to OVX+E and ORCH+E ($P<0.05$). For sample numbers, refer to the legend for Figure 1. Bivariate correlational analyses were used to determine the statistical significance of the relationship between tumor-induced mechanical allodynia and TRPV1 mRNA expression (B) in intact females and (C) in intact males. Significance is indicated with an asterisk ($P<0.05$)

Figure 4: JNJ-17203212, a TRPV1 antagonist, reduces tumor-induced hyperalgesia.

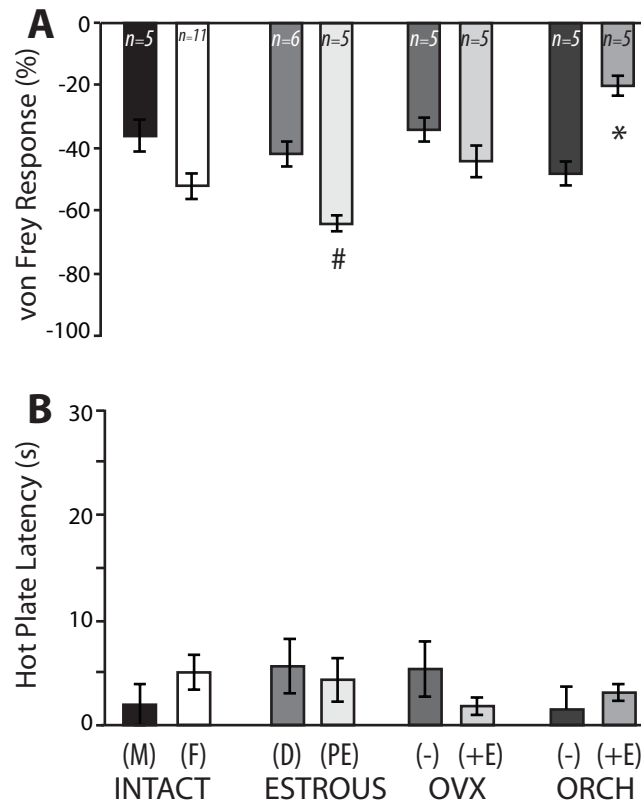


Figure 4: The effect of subcutaneous injection of JNJ-17203212 on mechanical allodynia (A) and thermal hyperalgesia (B) was assessed in all groups. Antinociceptive responses were measured by subtracting pre-treatment nociceptive responses from the post-treatment assessments. Data are expressed as the mean (\pm SEM) changes in percent response (%) to a von Frey fiber and in the hot plate thresholds (sec). Data were analyzed using a one-way ANOVA with Tukey HSD post hoc test. Hash tag (#) indicates females in proestrus/estrus phase of the estrous cycle are significant compared to intact males, diestrus females, OVX, OVX+E, and ORCH+E where significance indicated by an asterisk (*) indicates ORCH+E males are significant compared to intact females, OVX+E, and ORCH ($P < 0.05$).

Chapter 5

Summary and Conclusions

Summary

This thesis provides several important and novel findings related to tumor-induced nociception and the effects of sex steroid hormones (see Summary Table). First, the stage of the estrous cycle affects tumor-induced mechanical hyperalgesia with females in diestrus exhibiting reduced mechanical hyperalgesia compared to females in proestrus/estrus. Second, while it is known that TRPV1 receptor expression and activation is increased in DRG neurons of tumor-bearing males [59; 70; 97; 135; 153], we are the first to demonstrate that females also have an increase in TRPV1 receptor mRNA expression in DRG neurons innervating the hindpaw. Moreover, we are the first to show males and females differed in their tumor-induced expression of TRPA1 and TRPM8 receptor mRNA in DRG neurons. Third, using a known TRPV1 receptor antagonist, we were able to induce analgesia in tumor-bearing males and demonstrate that females' analgesic response is dependent upon estrogen. Moreover, using a known TRPM8 agonists, the present study is the first to examine and show an antinociceptive effect on tumor-induced pain.

Sex differences in tumor-induced mechanical allodynia manifested in our model of bone cancer pain with females in diestrus phase of the estrus cycle less sensitive to mechanical stimulation than females in the proestrus/estrus phase and males. When the cyclical effects of female sex steroid hormones were controlled with gonadectomy (OVX), OVX females have mechanical allodynia that were similar to females in the diestrus phase of the estrous cycle. When 17 β -estradiol was replaced (OVX+E), mechanical allodynia was greater in OVX+E which is similar to that observed in females in the proestrus/estrus phase of the estrous cycle. These results reveal that tumor-induced allodynia fluctuates with the phase of the estrous cycle. Moreover, our results indicate that female sex steroid hormones, specifically estradiol, enhances, rather than inhibits, tumor-induced mechanical allodynia in females.

In other models of pain, sex steroid hormones in males reduce nociception [54; 126]. Thus, we examined the effect of tumor-induced nociception in gonadectomized males (ORCH) whose circulating sex steroid hormones would be diminished. Consistent with previous findings, ORCH males have greater

tumor-induced mechanical allodynia compared to intact counterparts. 17β -estradiol replacement (ORCH+E) reduced mechanical allodynia similar to that observed in females with reduced sex steroid hormones (diestrus/OVX). This suggests that estrogen plays an opposite role in males and females with tumor-induced nociception. Furthermore, these findings suggest that male's circulating hormones are necessary for reduced tumor-induced mechanical allodynia and that estrogen plays a role in this phenomenon.

Sex differences in tumor-induced cold hyperalgesia manifested in our model of bone cancer pain with females becoming more sensitive than males. Although we show tumor-induced mechanical allodynia is sensitive to the effects of estrogen, cold-plate hyperalgesia was relatively insensitive to changes in estrogen. Although tumor-bearing females were more sensitive to the cold plate, cold-plate sensitivity was not attenuated by the estrous cycle or the addition of estrogen to gonadectomized mice.

TRPV1, TRPA1, and TRPM8 receptors transmit noxious thermal, mechanical and chemical sensitivities that contribute to pain hypersensitivity associated with peripheral inflammatory and neuropathic pain states [34; 109; 130]. In this thesis we show both males and females have an increase in TRPV1 mRNA in DRG neurons innervating the tumor-bearing hindpaw. Moreover, we found that the estrous cycle influences TRPV1 receptor mRNA expression as females in diestrus have lower TRPV1 receptor mRNA expression than those in proestrus/estrus, where the expression is increases nearly 5-fold. TRPV1 receptor is activated by mechanical stimulation in rodent models of inflammatory pain [91; 142]. In our model of bone cancer pain, tumor-induced mechanical allodynia was similar between males and females, and not surprisingly, TRPV1 receptor mRNA expression was also similar. However, when the phase of the estrous cycle was taken into account, tumor-induced mechanical allodynia was lower in females during diestrus, which paralleled TRPV1 receptor mRNA expression that is also significantly lower during diestrus. These findings would suggest the difference in tumor-induced mechanical allodynia during the phases

of the estrous cycle are due, in part, to the fluctuations in expression of the TRPV1 receptor expression.

TRPA1 receptor is considered a thermo-TRP that is gated by noxious cold [3; 76; 78] and has been shown to be involved in cold-induced nociceptor behavior in mice [67]. TRPM8 receptor is another thermo-TRP involved in both cooling-mediated analgesia and cold hypersensitivity after injury in rodent models of pain [7; 31; 42; 131]. In our model of bone cancer pain, males and females differed in their expression of cancer-induced changes in TRPA1 receptor and TRPM8 receptor expression, where intact males expressed more TRPA1 receptor and intact females express more TRPM8 receptor. Unlike the TRPV1 receptor, the phase of the estrous cycle did not affect the expression of TRPA1 receptor and TRPM8 receptor mRNA. Females were more sensitive to tumor-induced cold hyperalgesia than males. With TRPM8 receptor increasing in tumor-bearing females, this would suggest that TRPM8 receptor corresponds with tumor-induced cold hyperalgesia in females.

The use of TRP agonists and antagonists to abate peripheral and central neuropathic pain has been analyzed in rodent models of pain [108]. We examined the analgesic effects of a TRPV1 receptor-selective antagonist and a TRPM8 receptor-selective agonist on bone tumor-induced nociception. JNJ-17203212, a TRPV1 receptor-selective antagonist attenuates both ongoing and movement-evoked nocifensive behaviors in male mice with bone cancer pain [53]. In our model of fibrosarcoma bone cancer pain, antagonism of the TRPV1 receptor also attenuated mechanical allodynia in both males and females. The efficacy of JNJ-17203212 appeared to be estrogen-dependent as JNJ-17203212 reduced mechanical pain more during proestrus/estrus than during diestrus. Menthol, a TRPM8-selective agonist, is a natural compound of plant origin that is analgesic at low concentrations [38; 66; 136; 143]. To our knowledge, the present study is the first to show an antinociceptive effect of topical menthol on tumor-induced pain. The efficacy of menthol appears to be estrogen dependent as gonadectomized females (OVX) have less analgesia than females with 17 β -estradiol replacement. Taken together, our results suggests JNJ-17203212 and menthol are potentially novel pharmaceutical compounds that could be used for

the treatment of bone cancer pain. With that said, our work also suggest that the antinociceptive effect will depend on the hormonal status of the patient, which needs to be taken into account.

Conclusions

Using a well-established mouse model of bone cancer pain [141], and we hypothesized that that estrogen would potentiate cancer pain by altering tumor-induced transcription of TRP receptors and their function. We documented that sex differences exist in tumor-induced mechanical allodynia and cold-hyperalgesia. We also showed that estrogen influences tumor-induced sensitivity making females more sensitive to mechanical allodynia, whereas in males is makes them less sensitive. Conversely, estrogen did not effect thermal hyperalgesia, but instead the sex of the animal was most important with females having more tumor-induced cold sensitivity than males. We delineated that both males and females up-regulate TRPV1 receptor mRNA in lumbar DRGs, but differ in their expression of TRPM8 receptor and TRPA1 receptor mRNA with tumor-bearing females up-regulating TRPM8 receptor only. Moreover,

antagonists of TRPV1 receptor and agonists for TRPM8 receptor were effected by estrogen as females in proestrus/estrus phase of the estrous cycle or with estrogen replacement exhibited the greatest analgesia. Overall, we prove our hypothesis that estrogen potentiates cancer pain, but also show that it attenuates pain the males. We also prove that estrogen alters tumor-induced transcription of TRP receptor mRNA and alters the activity of selected TRPV1 receptor antagonist and TRPM8 receptor agonists. Our findings suggest that estrogen potentiates tumor-induced nociception through its effects on TRP receptors.

This work adds to the growing body of evidence that sex steroid hormones affect tumor-induced pain at the behavioral and the molecular level of pain signaling. Future models of pain and analgesic research should include both sexes and make note of the phases of the estrous cycle in females. The next steps that are required to build on this body of work include testing of other analgesic drugs to determine if there are sex differences or estrogen effects on drug efficacy in bone the treatment of bone cancer pain. It will also be important to test other types of cancer (pancreatic, ovarian, prostate, breast,

etc) to determine if sex hormones affect tumor-induced pain. Clearly more research is necessary since cancer, second only to heart disease in cause of death in Americans, has a nociceptive component that will effect many lives. Since we are just beginning to understand how sex hormones and sex differences play key roles in the generation and perception of pain, it is critical to incorporate sex difference as part of the basic research paradigm to uncover mechanisms of pain and analgesic drug efficacy.

Table: Summary of findings across experimental groups.

Tumor-Induced Nociception	Intact Males	ORCH	ORCH+E	Intact Females	OVX	OVX+E
Von Frey Fiber	<i>Allodynia</i>	<i>Allodynia</i>	Less Allodynia	<i>Allodynia</i>	Less Allodynia	<i>Allodynia</i>
Cold Plate Assay	Less Hyperalgesia	<i>Hyperalgesia</i>	<i>Hyperalgesia</i>	<i>Hyperalgesia</i>	<i>Hyperalgesia</i>	<i>Hyperalgesia</i>
Hot Plate Assay	<i>No Effect</i>	<i>No Effect</i>	<i>No Effect</i>	<i>No Effect</i>	<i>No Effect</i>	<i>No Effect</i>
Menthol-Induced Antinociception						
Von Frey Fiber	<i>Antinociception</i>	Reduced Antinociception	<i>Antinociception</i>	<i>Antinociception</i>	Reduced Antinociception	<i>Antinociception</i>
Cold Plate Assay	<i>Antinociception</i>	<i>Antinociception</i>	<i>Antinociception</i>	<i>Antinociception</i>	Reduced Antinociception	<i>Antinociception</i>
TRPV1 Antagonist-Induced Antinociception						
Von Frey Fiber	<i>Antinociception</i>	<i>Antinociception</i>	Reduced Antinociception	<i>Antinociception (Pro/estrus phase most significant)</i>	<i>Antinociception</i>	<i>Antinociception</i>
Hot Plate Assay	<i>No Effect</i>	<i>No Effect</i>	<i>No Effect</i>	<i>No Effect</i>	<i>No Effect</i>	<i>No Effect</i>
Tumor-Induced TRP mRNA						
TRPA1	<i>Increased</i>	<i>Increased</i>	<i>Increased</i>	Decreased	<i>Increased</i>	<i>Increased</i>
TRPM8	Decreased	<i>Increased</i>	<i>Increased</i>	<i>Increased</i>	<i>Increased</i>	<i>Increased</i>
TRPV1	<i>Increased</i>	<i>Increased</i>	<i>Increased</i>	Increased in Pro/Estrus Decreased in Diestrus	<i>Increased</i>	Decreased

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