Published in final edited form as:

Neuroscience. 2015 August 20; 301: 235–245. doi:10.1016/j.neuroscience.2015.06.009.

Colocalization of aromatase in spinal cord astrocytes: Differences in expression and relationship to mechanical and thermal hyperalgesia in murine models of a painful and a non-painful bone tumor

Elaine E O'Brien^a, Branden A Smeester^a, Kyle S Michlitsch^a, Jang-Hern Lee^b, and Alvin J Beitz^c

^aDepartment of Veterinary and Biomedical Sciences, College of Veterinary Medicine, University of Minnesota, St Paul, MN, USA

^bDepartment of Veterinary Physiology, College of Veterinary Medicine and Research Institute for Veterinary Science, Seoul National University, Seoul 151-742, Republic of Korea

^cDepartment of Veterinary and Biomedical Sciences, College of Veterinary Medicine, University of Minnesota, 1971 Commonwealth Ave, St. Paul, MN 55108

Abstract

While spinal cord astrocytes play a key role in the generation of cancer pain, there have been no studies that have examined the relationship of tumor-induced astrocyte activation and aromatase expression during the development of cancer pain. Here, we examined tumor-induced mechanical hyperalgesia and cold allodynia, and changes in GFAP and aromatase expression in murine models of painful and non-painful bone cancer. We demonstrate that implantation of fibrosarcoma cells, but not melanoma cells, produces robust mechanical hyperalgesia and cold allodynia in tumor-bearing mice compared to saline-injected controls. Secondly, this increase in mechanical hyperalgesia and cold allodynia is mirrored by significant increases in both spinal astrocyte activity and aromatase expression in the dorsal horn of fibrosarcoma-bearing mice. Importantly, we show that aromatase is only found within a subset of astrocytes and not in neurons in the lumbar spinal cord. Finally, administration of an aromatase inhibitor reduced tumor-induced hyperalgesia in fibrosarcoma-bearing animals. We conclude that a painful fibrosarcoma tumor induces a significant increase in spinal astrocyte activation and aromatase expression and that the up-regulation of aromatase plays a role in the development of bone tumor-induced hyperalgesia. Since spinal aromatase is also upregulated, but to a lesser extent, in non-painful melanoma bone tumors, it may also be neuroprotective and responsive to the changing tumor environment.

Correspondence: Alvin J. Beitz, Department of Veterinary and Biomedical Sciences, University of Minnesota, Room 205 Veterinary Medicine Bldg, 1971 Commonwealth Ave, St Paul, MN 55108, (Phone): 612-625-2595, beitz001@umn.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

Astrocytes; cancer pain; aromatase; letrozole; mechanical hyperalgesia; spinal cord

Introduction

Recently it has become evident that estradiol in males is responsible for a number of effects originally attributed to testosterone (de Ronde and de Jong, 2011). Recent work has shown that male rats exposed to acute foot shock have increased circulating estradiol (Lu et al., 2015). In addition to peripheral sources, estradiol is also synthesized in situ from testosterone by spinal tissue via the enzyme aromatase, where it appears to be biologically active only at the local tissue level (Simpson and Davis, 2001). In the quail, aromatase has been shown to be present in neurons in spinal cord laminae I-II. Chronic and systemic blockade of this enzyme with an aromatase inhibitor altered nociception within days (Evrard, 2006). Recently, aromatase was shown to be upregulated in the spinal cord of female rats with a central pain syndrome (Ghorbanpoor et al., 2014), but these investigators did not examine male animals. Localization of aromatase in the murine spinal cord and its potential role in chronic pain conditions including cancer pain has not yet been reported and is the topic of this report.

Studies with animal models of pain have suggested that the reaction of glia, including microglia and astrocytes, critically contributes to the development and maintenance of chronic pain. In particular, astrocyte activation in the spinal cord appears to be an important contributor to the chronic pain associated with inflammation (Ikeda et al., 2012), HIV (Shi et al., 2012), chemotherapy-induced neuropathy (Zhang et al., 2012, Ruiz-Medina et al., 2013) and tumor-induced pain (Geis et al., 2010, Yao et al., 2011, Ren et al., 2012). Activation may result in altered cell morphology, changes in receptor expression, or release of factors by glial cells, which ultimately enhance nociceptive transmission (Ren and Dubner, 2008). The literature remains controversial regarding whether astrocytes play a critical role in the development of cancer pain (Ren et al., 2012; Ducourneau et al., 2014, Hironaka et al., 2014). Moreover there are no studies in the literature that have examined whether differential expression of spinal aromatase is associated with the development or maintenance of cancer pain.

The major goal of this study was to examine the relationships among tumor-induced nociception, astrocyte activation and aromatase expression in the spinal cord in murine models of painful and non-painful bone cancer. In this initial study we examined male mice, since the only other study on aromatase in the mammalian spinal cord focused on female rats (Ghorbanpoor et al., 2014) and since as stated above estradiol in males is responsible for a number of effects originally attributed to testosterone. We hypothesized that: 1) mechanical and cold hyperalgesia would be evident in mice with the fibrosarcoma bone tumor, but not in mice with a melanoma bone tumor; 2) astrocyte activation would be greater in fibrosarcoma-bearing animals compared to non-painful melanoma-bearing animals; 3) aromatase would be upregulated in mice with the painful fibrosarcoma tumor

and not in mice with the melanoma tumor; and that 4) giving an aromatase inhibitor would inhibit mechanical hyperalgesia by reducing aromatase expression.

2. Experimental Procedures

2.1 Animals

A total of 81 male C3H animals that are syngeneic to fibrosarcoma cells and male B6C3F1/Cr (B6C3) male mice (an F1 cross between C3H/He and C57BL/6 strains) that are syngeneic to G3.26 melanoma cells were used for all experiments. All mice were 6-8 weeks old and were obtained from the National Cancer Institute (Bethesda, MD). Mice were housed in small conventional boxes in a temperature and humidity-controlled environment and maintained on a 12 h light/dark cycle with *ad libitum* access to food and water. All experiments were approved by the Institutional Animal Care and Use Committee at the University of Minnesota.

2.2 Cell culture and preparation for implantation

Fibrosarcoma NCTC 2472 cells were obtained from the American Cell Culture Collection (Rockville, MD) and were maintained in NCTC 135 Medium (Sigma-Aldrich, Munich, Germany). Melanoma G3.26 cells were obtained from Dr. Christopher W. Stackpole (New York Medical College, Valhalla, NY) and maintained in alpha MEM (Sigma-Aldrich, St. Louis, MO) with 10% FBS as previously described (Wacnik et al., 2001). MB-MDA 231 cells were a gift from Dr. Laura Mauro at the University of Minnesota. These cells were maintained in DMEM media (Gibco, Life technologies, Grand Island, NY), 10% fetal bovine serum and 1% penicillin-streptomycin (Life technologies, Grand Island, NY). Cells were first washed with PBS, trypsinized, pelleted and suspended in 5 mL of PBS for quantification using a hemacytometer. Following counting, all cells were re-pelleted and re-suspended in a volume of PBS for a final concentration of 2×10^5 fibrosarcoma cells per 10 μ L or 1.5×10^5 melanoma cells per 10 μ L. Cells were kept on ice and vortexed shortly before tumor implantation.

2.3 Tumor implantation

Implantation into the calcaneus bone of the hind paw was performed as previously described (Wacnik et al., 2001, Smeester et al., 2014). Briefly, mice were placed in an enclosed plexi-glas chamber and initially anesthetized with 3% isoflurane/3 L oxygen. Upon successful anesthetization, the flow rate was adjusted to a maintenance level of 2% isoflurane/1.5 L oxygen for the remainder of the implantation procedure. Cells were injected unilaterally into the left heel using a 29 gauge, sterile single-use needle attached to a 0.3 mL insulin syringe (Becton Dickinson, Franklin Lakes, NJ) to manually bore into the hind paw calcaneus bone. Control mice underwent an identical procedure with the exception that they received injection of saline rather than tumor cells. Following implantation, animals were returned to their home cages and recovered on a heating pad. Animals showing any signs of dysfunction (e.g. problems with ambulation, lethargy or excessive bleeding) were removed from the study. This occurred in less than 1% of the animals used in this study.

2.4 Mechanical hyperalgesia

Tumor-induced mechanical hyperalgesia was tested using von Frey filaments (#3.61- C3H mice and #1.65 - B6C3) (Smeester et al., 2012, Smeester et al., 2013, Smeester et al., 2014). These filaments produce forces of 400 mg and 8 mg respectively. Animals were placed under clear glass cups on a wire grid and allowed to acclimate for 30 min. Starting with the right hind paw, the numbers of positive responses out of a total of 10 applications were recorded. Baseline von Frey measurements were obtained prior to tumor implantation or saline control injection into the calcaneus. Subsequent von Frey measurements were on post-implantation (PID) days 3, 7, 10, and 14. Behavioral assessments were conducted at approximately the same time each day. The investigator performing the von Frey testing was blinded to the experimental condition of the animals being tested. As the tumor grew the investigator was no longer blinded to the experimental condition, but the analysis of the data was performed by another researcher blinded to the experimental conditions of the animals.

2.5 Cold plate hypersensitivity

A modified version of the cold plate procedure reported by Allchorne and colleagues (Allchorne et al., 2005) was utilized for all cold plate testing. Mice were placed on top of a Peltier cold plate (model LHP-1200CPV, TECA Corp., Chicago, IL) in an enclosed container (20×16×25 cm³) maintained at 4 ± 0.1°C where the number of licking or rapid shaking behaviors of the hind paw was recorded and quantified by trained observers (number of behaviors). A maximum cut off time of 2 minutes was observed for all cold plate testing to prevent tissue damage at lower temperatures. Each mouse was only tested once on any given test day to avoid any possible anesthetic or tissue damage effects that could be produced by repeated exposure to the cold surface.

2.6 Transcardiac perfusion

Animals were deeply anesthetized with sodium pentobarbital (Nembutal, Ovation Pharmaceuticals, Inc., Deerfield, IL) and transcardiac perfusion was performed as previously described (Smeester et al., 2013). Briefly, follow atrial puncture using a 21-gauge butterfly catheter (Terumo Medical Corporation, Somerset, NJ), 15 mL ice cold PBS followed by 30 mL of 10% neutral buffered formalin (NBF) was administered via peristaltic pump at a rate of 3 mL/min. Following perfusion, tissues were prepared for immunohistochemical (IHC) study or western blot (WB). Spinal columns for IHC were excised and post-fixed in 10% NBF overnight at 4°C and cryoprotected for an additional 24 h prior to tissue sectioning. Spinal cords for WB were obtained by pushing the spinal cord out of the vertebral column with PBS in a 20 mL syringe attached to a 17g needle; cerebellum was excised from the skull. The lumbar region of the spinal cords and the cerebellum were flash frozen in liquid nitrogen and stored at -80°C until ready to use.

2.7 Antibody generation

Rabbit anti-mouse aromatase antibodies were developed in our laboratory in collaboration with Proteintech Group, Inc (Chicago, IL). The murine aromatase peptide sequences LYRKYERSVKDLKDEI and PNEDRHLVEIIFSPRNSDKY* were conjugated to KLH and used to generate mouse specific aromatase antibodies. Rabbit antisera were then subjected to

antigen-specific affinity purification and subsequently evaluated using a western blot analysis to determine antibody specificity only.

*LYRKYERSVKDLKDEI =

CTCTACAGAAAGTATGAACGATCCGTCAAGGACTTGAAAGACGAGATAGCTGTT
TTG

PNEDRHLVEIIFSPRNSDKY =

CCAAATGAGGACAGGCACCTTGTGGAAATAATTTCTCTCCAAGGAATTCAGAC
AAGTAC

2.8 Immunohistochemistry

Spinal cords were sectioned and double immunostained with specific antibodies for aromatase, astrocytes and neurons. Lumbar regions (L4-L5) of spinal cords were sliced at 40 μ m on a sliding microtome. Sections were incubated in 2% goat serum in 0.3% TritonX for one hour at room temperature. Following incubation, (a) rabbit anti-aromatase (1:10,000, Proteintech Group, Inc., Chicago, IL) and (b) rat anti-GFAP (1:1,000, catalog #13-0300, Invitrogen, Carlsbad, CA), or anti-aromatase with (c) mouse anti-NeuN (1:1,000, gift from Dr. Lucy Vulchanova-Hart, University of Minnesota) were added to sections overnight at 4°C. Sections were then washed 5X with PBS over a 25-minute period. Following washing, secondary antibodies were applied: (a) anti-rabbit conjugated to cyanine 5 (Cy5), (b) anti-rat conjugated to cyanine 3 (Cy3) and (c) anti-mouse conjugated to cyanine 3 (Cy3) (1:400 all secondaries, Jackson ImmunoResearch, West Grove, PA) for 2 hours at room temperature. Sections were again washed with PBS, rinsed in molecular water, mounted on gelatin-coated slides and cover-slipped using Prolong gold mounting medium (Invitrogen, Carlsbad, CA). Immunohistochemical controls consisted of: 1) no primary antibody, 2) primary, but no secondary antibody and 3) an absorption control in which the primary antibody was first pre-incubated with immunogen (10:1 molar ratio of antigen to antibody, see section 2.7) prior to tissue incubation. Additionally, to control for staining intensity variance, parallel sections of naive C3H and/or B6C3 mouse spinal cord served as standards for immunofluorescence measurements.

2.9 Western blot

Western blotting was used to determine antibody specificity. Lumbar spinal cord segments or cerebellum were homogenized in 50 μ L PBS/1X Complete, Mini protease inhibitor cocktail (Roche, Mannheim, Germany). Protein concentration was determined using a BCA Protein Assay (Pierce, Rockford, IL).

Samples containing 30 μ g of protein were heated at 80°C for 5 minutes in loading buffer, cooled on ice for 5 minutes and loaded into each well. SDS-PAGE was performed on a 10% Mini-Protean TGX precast gel (Bio-Rad, Richmond, CA) at 150 volts for one hour, transferred onto a nitrocellulose membrane and then blocked with Odyssey Blocking Buffer for 2 hours at room temperature. Blots were incubated with (a) anti-aromatase (1:3,000, Proteintech Group, Inc., Chicago, IL) and (b) anti-beta actin (1:1,000, Sigma, St. Louis, MO) in 12.5 mL of blocking buffer overnight at 4°C.

Membranes were incubated with 30 mL of blocking buffer containing secondary antibodies (a) goat anti-rabbit 680 and (b) goat anti-mouse 800 (1:10,000 both secondaries, Li-cor, Lincoln, NE) for one hour at room temperature. MD-MDA 231 breast cancer cells served as a negative control while cerebellum served as a positive control for aromatase (Mirzatoni et al., 2010). The resulting western blot was imaged and analyzed using the Li-Cor Odyssey infrared imaging system.

2.10 Drug treatment

The aromatase inhibitor, letrozole (catalog #L6545, Sigma-Aldrich, St Louis, MO) was daily administered subcutaneously (s.c.) (10 µg/d in 0.1 mL 0.3% hydropropylcellulose; HPC) beginning on post-implantation day 7 (PID 7) for a total of 7 days. Similarly, control animals received a daily subcutaneous injection of vehicle (0.1 mL 0.3% HPC) over the same time course. This optimized dose of letrozole was selected based on a previous report by Lu et al. that found this dose to be the most effective dose when examining the effects of aromatase inhibitors on the growth of mammary tumors (Lu et al., 1999).

2.11 Imaging

All imaging was performed on a Nikon Eclipse 80i fluorescent microscope and on an Olympus FLUOVIEW 1000 confocal microscope. Co-localization images were taken at 200X and 400X magnification, while representative images of spinal cord immunostaining were taken at 100X or 200X magnification as indicated.

2.12 Quantification of aromatase and GFAP immunoreactivity (-ir)

A total of 10 randomly selected sections (from the L4 and L5 spinal cord segments) per animal per group were scanned for the presence of aromatase and GFAP immunoreactivity (-ir) using fluorescence microscopy (Nikon, Melville, NY). The investigator scanning the images and performing the analysis was blinded to the treatment groups. Digital images of aromatase and GFAP staining were captured using Nikon ACT-1 software (Nikon, Melville, NY). The brightness level was normalized per animal and within groups using this software. GFAP was quantified on both the ipsilateral and contralateral sides in laminae I-II, III-IV, V-VI and X in both fibrosarcoma and melanoma-bearing animals as well as controls. Aromatase-ir was quantified in total for both tumor types throughout all laminae and specifically in fibrosarcoma animals. A quantitative approach to cell morphometric density measurement was conducted for all GFAP-ir using ImageJ software (NIH, Bethesda, MD) due to the robustness of staining. Consistency was insured throughout all subsequent density measurements by setting threshold values identical in all animals and groups analyzed. Average area pixel count for GFAP-ir was calculated following thresholding of respective gray-scale images to reduce the contribution of nonspecific background signal. The immunopositive pixels were again selected and a threshold pixel count was acquired for each channel. Unlike GFAP-ir density measurements, aromatase-ir positive cells were manually counted using ImageJ in all laminae sampled. In addition to examining tumor-induced changes in aromatase in the L4-L5 spinal cord, potential tumor-induced changes in aromatase in the mid-thoracic (T6-T7) and cervical (C6-C7) regions of the spinal cord were also analyzed.

2.13 Statistical analyses

Complete statistical analyses of all data sets were carried out. Comparisons between groups were performed using a two-way ANOVA with post hoc comparisons using Bonferroni's method. For single time point comparisons between groups or within a group, an unpaired Student's t-test was employed. Data was analyzed and graphed using Prism 5.0 (GraphPad Software, La Jolla, CA). The level of significance was set at $p < 0.05$.

3. Results

3.1 Murine spinal cord tissue expresses aromatase

Our rabbit-antimouse aromatase antibody was evaluated for specificity prior to further immunohistochemical (IHC) experiments. Western blot shows that lower lumbar spinal cord tissue from naïve animals expresses aromatase (Fig. 1; lanes 3, 4 and 5 - naïve). A band corresponding to the expected molecular weight of aromatase was detected at 55 kDa. Similarly, aromatase positive staining occurred in cerebellar control tissue (lane 1), but not in aromatase negative MD-MDA 231 control cells (lane 2). All subsequent IHC experiments were performed using this antibody.

3.2 Localization of aromatase in astrocytes, but not neurons

Double-labeling immunohistochemical (IHC) studies were performed to determine cell-type specificity in the mouse spinal cord. Co-localization was observed in GFAP-expressing, aromatase-positive cells (Fig. 2A, right panel 'merged', black arrows). Conversely there was no co-localization of aromatase with NeuN (Fig. 2B) indicating that neurons in the mouse spinal cord do not express aromatase as they do in the quail. While aromatase was co-localized with GFAP in astrocytes, not all astrocytes contained aromatase. Thus, aromatase appears to be found in a sub-set of spinal cord astrocytes..

3.3 Painful fibrosarcoma induces mechanical hyperalgesia

Following tumor cell implantation, robust development of mechanical hyperalgesia was evident in fibrosarcoma mice as early as post-implantation day 7 (PID 7) and continued for the length of the experiment when compared with saline controls (Fig. 3; **** $p < 0.0001$). Conversely, implantation of melanoma cells into the calcaneus bone of B6C3 mice did not induce mechanical hyperalgesia at any of the time points tested (Fig. 3, $p > 0.05$).

3.4 Cold hypersensitivity induced by fibrosarcoma only

The numbers of spontaneous behaviors were recorded at baseline (BL) prior to injection of tumor cells or saline into the calcaneus and then following implantation of fibrosarcoma (A) or melanoma (B) cells at 7, 10 and 14 days post-injection (PID) (Fig. 4A-B). Changes in the number of spontaneous behaviors were observed in fibrosarcoma-bearing animals when exposed to a cold plate for 2 min at 4°C at all time points tested as compared to saline controls (Fig. 4A; $p < ****0.0001$). No significant changes were observed in melanoma-bearing mice when compared to their controls (Fig. 4B; $p > 0.05$).

3.5 Distribution of activated astrocytes in the spinal cord of fibrosarcoma mice

Lower lumbar (laminae I-II, III-IV, V-VI, X) astrocyte expression was quantified in fibrosarcoma and melanoma-bearing animals at PID 14, the time point at which maximal mechanical hyperalgesia was observed (Fig. 3). Increased GFAP-ir on PID 14 in fibrosarcoma animals was observed in all laminae analyzed as compared to their saline controls (Fig. 6A; ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). The largest fold difference occurred in laminae V and VI, where GFAP staining increased 14 fold compared to saline injected control mice. No differences in GFAP-ir were seen in any laminae in melanoma animals as compared to their saline controls (images Figs. 5A-B and graph 6B; $p > 0.05$). Additionally, there were no significant differences in tumor-induced astrocyte activation on the contralateral side of the lumbar spinal cord in fibrosarcoma-injected mice (data not shown).

3.6 Tumor-induced aromatase activation in the lumbar spinal cord

Sections positive for GFAP from both fibrosarcoma- and melanoma-injected mice were double-stained for the presence of aromatase and aromatase immunostaining was only found in a subset of astrocytes. All immunopositive aromatase cells were counted and expressed as a total count in fibrosarcoma (A) and melanoma (B) -bearing animals in laminae I-X of the dorsal horn in the lumbar region on post-implantation day 14 (Fig. 7). Note the number of aromatase positive cells in spinal cord sections of both saline-injected and melanoma B6C3 (Figs. 5C-D) mice were greater than that observed in C3H/He saline-injected and fibrosarcoma-bearing animals (Fig. 2A). Tumor-induced increases in aromatase-ir cell counts were observed in both tumor types as compared to saline injected control mice (Fig. 7A-B; **** $p < 0.0001$). In this regard both tumor types displayed an increase in aromatase-ir in the dorsal horn, however; fibrosarcoma animals had a significantly larger fold change in aromatase-ir when compared to melanoma-bearing animals (Fig. 7C). Due to this significant change in fibrosarcoma mice, aromatase-ir cells were further quantified in specific laminae of the dorsal horn (laminae I-II, III-IV, V-VI and X) only in fibrosarcoma tumor-bearing mice (Fig. 7D). When compared to controls, fibrosarcoma animals had increased aromatase positive cell counts in all laminae (Fig. 7D, * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$). Finally, there were no increases in aromatase staining in the dorsal horn of the thoracic and cervical segments of the spinal cord in either fibrosarcoma or melanoma injected mice.

3.7 Letrozole inhibits mechanical hyperalgesia and aromatase expression

To determine whether an aromatase inhibitor could decrease the observed mechanical hyperalgesia and counter tumor-induced increases in aromatase, Letrozole was given subcutaneously (10 $\mu\text{g/d}$ in 0.1 mL 0.3% HPC) beginning on post-implantation (PID) 7 for a total of 7 days. Although not quantitated, GFAP-ir appeared to be reduced in letrozole-treated animals (Fig. 8A v. B). Mechanical von Frey scores (C) and aromatase expression in laminae I-II, III-IV, V-VI and X (D) were evaluated on PID 14 (Fig. 8). Letrozole significantly reduced tumor-induced mechanical hyperalgesia and aromatase positive cell counts in laminae I-II on PID 14 as compared to controls (Fig. 8C-D; * $p < 0.05$, **** $p < 0.0001$).

Discussion

Bone cancer pain is a complex phenomenon in which patients suffer from background pain, spontaneous pain, and evoked pain (Laird et al., 2011). While the central and peripheral mechanisms responsible for these various types of cancer associated pain are still under investigation, it is clear the spinal cord glial cells play a major role (Yao et al., 2011), but apparently not in all types of cancer pain (Ducourneau et al., 2014, Hironaka et al., 2014). In this regard Ducourneau et al. (Ducourneau et al., 2014) showed no increase in GFAP in the spinal cord in a rat cancer pain model in which MRMT-1 mammary gland carcinoma cells were injected into the tibia, while Wang et al., (Wang et al., 2012) showed an increase in GFAP in the spinal cord in a rat model in which Walker 256 mammary gland carcinoma cells were injected into the tibia. Astrocyte activation has been shown to increase in bone cancer mice (Pevida et al., 2014) and both astrocytes and microglia have been shown to contribute to bone cancer pain in a rat model (Mao-Ying et al., 2012), but hyperactivation of these cells does not influence the maintenance of cancer pain hypersensitivity (Zhang et al., 2005). Finally, it has been recently reported that CXCL12 released from astrocytes acts on CXCR4 expressing microglia and astrocytes to activate them and contributes to the development of cancer pain (Hu et al., 2015). Because there is still controversy regarding the role of astrocytes in cancer pain and because there are no studies that have examined the potential role of spinal cord aromatase in cancer pain, we examined both astrocyte and aromatase expression in both a painful and nonpainful model of hindpaw bone cancer pain. Here we demonstrate that a bone tumor fibrosarcoma, but not a non-painful bone tumor melanoma, produces both robust mechanical hyperalgesia and thermal allodynia and that this increase in nociception is mirrored by a significant increase in astrocyte activation in the dorsal horn of fibrosarcoma mice, while no increase is observed in melanoma injected mice. Our results support previous studies showing that bone tumor pain is associated with an increase in astrocyte activation in the spinal cord dorsal horn (Schwei et al., 1999, Zhang et al., 2005, Wang et al., 2012, Xu et al., 2014) and extend these findings by demonstrating that a non-painful tumor does not cause astrocyte activation in the spinal cord.

With respect to specific laminae in the dorsal horn, we show that in fibrosarcoma mice, the largest fold increase in astrocyte activation occurs in laminae V/VI (14 fold increase compared to control mice) which correlates very well with the findings of Schwei et al. (Schwei et al., 1999). Moreover we found that GFAP immunostaining increased 3-fold in both laminae III/IV and laminae X. The increase in laminae X is interesting since lamina X, which surrounds the central canal, represents a unique sexually dimorphic region that is a convergence site for somatic and visceral afferent inputs including nociceptive information (Phelan and Newton, 2000). Injection of formalin into the hind paw causes a significant increase in Fos expression in lamina X and thus, it is perhaps not surprising that a hind paw tumor also activates this region of the spinal cord gray matter (Kaneko et al., 2000). Some neurons in lamina X conduct nociceptive information to the cerebellum, which serves to adjust motor programs in response to pain and injury (Huma et al., 2015) and this may be one of the important functions of this region in response to ongoing cancer pain. Recent work has also shown that lamina X contains an abundance of GABAergic neurons that modulate preganglionic sympathetic neurons (Deuchars, 2015). Whether neurons in lamina

X in the lumbar spinal cord segments influence preganglionic neurons remains to be determined, but it is interesting to speculate that cancer pain could modulate autonomic activity via this pathway.

Estrogens are synthesized from androgens by aromatase, a P₄₅₀ enzyme traditionally associated with discrete neuronal populations in the brain of vertebrates (Roselli and Resko, 1997, Carswell et al., 2005). It is clear that while there are sources of variability and inconsistent results in the literature regarding the effect of estrogen on nociception, the prevailing evidence supports the presence of significant sex hormonal influences on nociceptive processing (Fillingim and Ness, 2000). Initial studies reported no aromatase activity found in the spinal cord of rats (MacLusky et al., 1987), however; more recent work from Evrard and colleagues showed aromatase expression in neurons located in the dorsal horn of quail (Evrard, 2006). While subsequent physiological and pharmacological studies assumed aromatase was present in neurons of the mammalian spinal cord (see for example (Zhong et al., 2010), a recent report by Ghorbanpoor et al. (Ghorbanpoor et al., 2014) challenged this view by reporting aromatase in spinal cord glial cells of rats following spinal cord injury. Consistent with the findings of Ghorbanpoor and colleagues, using an antibody specific for the mouse aromatase sequence in combination with an antibody specific for GFAP, we demonstrate that aromatase expression in mouse spinal cord occurs in a sub set of astrocytes within the dorsal horn and not in neurons. This is consistent with other studies in brain that have demonstrated that aromatase is also localized to astrocytes; particularly reactive astrocytes following brain injury (Garcia-Segura et al., 1999, Gatson et al., 2011). Everard and Balthazart (Evrard and Balthazart, 2004) suggested that aromatase is involved in the regulation of thermal pain in quail based on experiments showing that treatment with a non-steroidal aromatase inhibitor increased thermal withdrawal latency (reduced thermal nociception). Conversely Ghorbanpoor et al. (Ghorbanpoor et al., 2014) reported that aromatase inhibition enhanced mechanical allodynia in both hind paws in a model of spinal cord injury-induced central pain syndrome. To test if aromatase is involved in mechanical hyperalgesia in our murine cancer model, we injected the non-steroidal aromatase inhibitor, letrozole in male fibrosarcoma mice. We observed a significant decrease in tumor-induced mechanical hyperalgesia scores following 7 straight daily subcutaneous injections of letrozole (Fig. 7A). Additionally, we observed a corresponding decrease in tumor-induced immunoreactive aromatase cells in letrozole treated mice with the greatest reduction occurring in laminae I-II (Fig. 7B). This would suggest that aromatase plays a role in tumor nociception by increasing estrogen locally in the spinal cord and is consistent with a report showing that estrogen acutely facilitates nociceptive transmission in the spinal cord via activation of membrane-bound estrogen receptors (Zhang et al., 2012). Our results are consistent with previous studies showing that estrogen is pronociceptive. In this regard Ralya and McCarson (Ralya and McCarson, 2014) showed that a surge in serum estrogen can produce a stimulus-intensity-dependent increase in inflammation-evoked nociceptive behavior, which is consistent with the findings of Lu and coworkers (Lu et al., 2012) showing that systemic application of estradiol, as well as 17 β -estradiol (E₂), increases hyperalgesia in CFA-induced chronic pain. In addition, (Zhang et al., 2012) showed that spinal administration of E₂ produces mechanical allodynia and thermal hyperalgesia in both male and female rats confirming the local spinal effect of E₂, while Cao et al. (Cao et al.,

2014) demonstrated that spinal E₂ facilitates the visceromotor response to colorectal distention.

Since spinal aromatase expression was also increased in non-painful (melanoma) tumor mice, albeit to a lesser extent than fibrosarcoma mice, it appears that the upregulation of aromatase may also be associated with the presence of the peripheral tumor or the tumor microenvironment. This upregulation of dorsal horn aromatase in the nonpainful melanoma mice may be associated with a neuroprotective response as has been demonstrated in the hippocampus (Garcia-Ovejero et al., 2005), cerebellum (Mirzamani et al., 2010) and cerebral cortex (Carswell et al., 2005). The majority of studies to date have focus on the effects of painful tumors on changes in spinal cord gene and protein expression as well as on neuronal and glial activation. Our results suggest that the presence of a non-painful tumor can also alter spinal cord protein expression, the enzyme aromatase in this case. Such changes were not evident in control mice injected with saline instead of melanoma cells.

In conclusion, our results show that a painful fibrosarcoma bone tumor, but not a non-painful melanoma bone tumor, upregulates GFAP, a marker of spinal cord astrocytes, in all dorsal horn laminae with the largest fold increase occurring in laminae V/VI. Importantly aromatase is upregulated in a sub-set of these activated astrocytes, which is important in regulating local estrogen concentrations in the spinal cord. Inhibition of aromatase with letrozole reduces tumor-induced mechanical hyperalgesia indicating that aromatase may contribute to tumor-induced nociception. The fact that aromatase, but not GFAP expression, is also increased in the spinal cord dorsal horn of mice implanted with a non-painful melanoma tumor, but to a lesser extent than in fibrosarcoma mice, further suggests that the tumor site is capable of up-regulating proteins in spinal cord astrocytes that are not related to tumor-induced nociception.

Acknowledgements

This work was supported by NIH grant CA084233 and Minnesota AES grant MIN-63-071.

References

- Allchorne AJ, Broom DC, Woolf CJ. Detection of cold pain, cold allodynia and cold hyperalgesia in freely behaving rats. *Molecular pain*. 2005; 1:36. [PubMed: 16354295]
- Cao DY, Bai G, Ji Y, Traub RJ. Epigenetic upregulation of metabotropic glutamate receptor 2 in the spinal cord attenuates oestrogen-induced visceral hypersensitivity. *Gut*. 2014
- Carswell HV, Dominiczak AF, Garcia-Segura LM, Harada N, Hutchison JB, Macrae IM. Brain aromatase expression after experimental stroke: topography and time course. *The Journal of steroid biochemistry and molecular biology*. 2005; 96:89–91. [PubMed: 15896953]
- de Ronde W, de Jong FH. Aromatase inhibitors in men: effects and therapeutic options. *Reproductive biology and endocrinology : RB&E*. 2011; 9:93. [PubMed: 21693046]
- Deuchars SA. How sympathetic are your spinal cord circuits? *Experimental physiology*. 2015; 100:365–371. [PubMed: 25655449]
- Ducourneau VR, Dolique T, Hachem-Delaunay S, Miraucourt LS, Amadio A, Blaszczyk L, Jacquot F, Ly J, Devoize L, Olier SH, Dalle R, Mothet JP, Nagy F, Fenelon VS, Voisin DL. Cancer pain is not necessarily correlated with spinal overexpression of reactive glia markers. *Pain*. 2014; 155:275–291. [PubMed: 24120461]

- Evrard HC. Estrogen synthesis in the spinal dorsal horn: a new central mechanism for the hormonal regulation of pain. *American journal of physiology Regulatory, integrative and comparative physiology*. 2006; 291:R291–299.
- Evrard HC, Balthazart J. Rapid regulation of pain by estrogens synthesized in spinal dorsal horn neurons. *The Journal of neuroscience*. 2004; 24:7225–7229. [PubMed: 15317848]
- Fillingim RB, Ness TJ. Sex-related hormonal influences on pain and analgesic responses. *Neuroscience and biobehavioral reviews*. 2000; 24:485–501. [PubMed: 10817845]
- Garcia-Ovejero D, Azcoitia I, DonCarlos LL, Melcangi RC, Garcia-Segura LM. Glia-neuron crosstalk in the neuroprotective mechanisms of sex steroid hormones. *Brain research Brain research reviews*. 2005; 48:273–286. [PubMed: 15850667]
- Garcia-Segura LM, Wozniak A, Azcoitia I, Rodriguez JR, Hutchison RE, Hutchison JB. Aromatase expression by astrocytes after brain injury: implications for local estrogen formation in brain repair. *Neuroscience*. 1999; 89:567–578. [PubMed: 10077336]
- Gatson J, Simpkins J, Yi K, Idris A, Minei J, Wigginton J. Aromatase is increased in astrocytes in the presence of elevated pressure. *Endocrinology*. 2011; 152:207–213. [PubMed: 21047944]
- Geis C, Graulich M, Wissmann A, Hagenacker T, Thomale J, Sommer C, Schafers M. Evoked pain behavior and spinal glia activation is dependent on tumor necrosis factor receptor 1 and 2 in a mouse model of bone cancer pain. *Neuroscience*. 2010; 169:463–474. [PubMed: 20417692]
- Ghorbanpoor S, Garcia-Segura LM, Haeri-Rohani A, Khodagholi F, Jorjani M. Aromatase inhibition exacerbates pain and reactive gliosis in the dorsal horn of the spinal cord of female rats caused by spinothalamic tract injury. *Endocrinology*. 2014; 155:4341–4355. [PubMed: 25105782]
- Hironaka K, Ozaki N, Hattori H, Nagamine K, Nakashima H, Ueda M, Sugiura Y. Involvement of glial activation in trigeminal ganglion in a rat model of lower gingival cancer pain. *Nagoya journal of medical science*. 2014; 76:323–332. [PubMed: 25741041]
- Hu XM, Liu YN, Zhang HL, Cao SB, Zhang T, Chen LP, Shen W. CXCL12/CXCR4 chemokine signaling in spinal glia induces pain hypersensitivity through MAPKs-mediated neuroinflammation in bone cancer rats. *Journal of neurochemistry*. 2015; 132:452–463. [PubMed: 25393328]
- Huma Z, Ireland K, Maxwell DJ. The spino-bulbar-cerebellar pathway: Activation of neurons projecting to the lateral reticular nucleus in the rat in response to noxious mechanical stimuli. *Neuroscience letters*. 2015; 591:197–201. [PubMed: 25711799]
- Ikeda H, Kiritoshi T, Murase K. Contribution of microglia and astrocytes to the central sensitization, inflammatory and neuropathic pain in the juvenile rat. *Molecular pain*. 2012; 8:43. [PubMed: 22703840]
- Kaneko M, Mestre C, Sanchez EH, Hammond DL. Intrathecally administered gabapentin inhibits formalin-evoked nociception and the expression of Fos-like immunoreactivity in the spinal cord of the rat. *The Journal of pharmacology and experimental therapeutics*. 2000; 292:743–751. [PubMed: 10640314]
- Laird BJ, Walley J, Murray GD, Clausen E, Colvin LA, Fallon MT. Characterization of cancer-induced bone pain: an exploratory study. *Supportive care in cancer : official journal of the Multinational Association of Supportive Care in Cancer*. 2011; 19:1393–1401. [PubMed: 20680354]
- Lu J, Wu XY, Zhu QB, Li J, Shi LG, Wu JL, Zhang QJ, Huang ML, Bao AM. Sex differences in the stress response in SD rats. *Behavioural brain research*. 2015; 284:231–237. [PubMed: 25687843]
- Lu Q, Liu Y, Long BJ, Grigoryev D, Gimbel M, Brodie A. The effect of combining aromatase inhibitors with antiestrogens on tumor growth in a nude mouse model for breast cancer. *Breast cancer research and treatment*. 1999; 57:183–192. [PubMed: 10598045]
- Lu Y, Li Z, Li HJ, Du D, Wang LP, Yu LH, Burnstock G, Chen A, Ma B. A comparative study of the effect of 17beta-estradiol and estradiol on peripheral pain behavior in rats. *Steroids*. 2012; 77:241–249. [PubMed: 22198527]
- MacLusky N, Clark C, Shanabrough M, Naftolin F. Metabolism and binding of androgens in the spinal cord of the rat. *Brain research*. 1987; 422:83–91. [PubMed: 3499956]

- Mao-Ying QL, Wang XW, Yang CJ, Li X, Mi WL, Wu GC, Wang YQ. Robust spinal neuroinflammation mediates mechanical allodynia in Walker 256 induced bone cancer rats. *Molecular brain*. 2012; 5:16. [PubMed: 22607655]
- Mirzaton A, Spence RD, Naranjo KC, Saldanha CJ, Schlenger BA. Injury-induced regulation of steroidogenic gene expression in the cerebellum. *Journal of neurotrauma*. 2010; 27:1875–1882. [PubMed: 20925573]
- Pevida M, Gonzalez-Rodriguez S, Lastra A, Garcia-Suarez O, Hidalgo A, Menendez L, Baamonde A. Involvement of spinal chemokine CCL2 in the hyperalgesia evoked by bone cancer in mice: a role for astroglia and microglia. *Cellular and molecular neurobiology*. 2014; 34:143–156. [PubMed: 24122510]
- Phelan KD, Newton BW. Intracellular recording of lamina X neurons in a horizontal slice preparation of rat lumbar spinal cord. *Journal of neuroscience methods*. 2000; 100:145–150. [PubMed: 11040377]
- Ralya A, McCarron KE. Acute estrogen surge enhances inflammatory nociception without altering spinal Fos expression. *Neuroscience letters*. 2014; 575:91–95. [PubMed: 24861514]
- Ren BX, Gu XP, Zheng YG, Liu CL, Wang D, Sun YE, Ma ZL. Intrathecal injection of metabotropic glutamate receptor subtype 3 and 5 agonist/antagonist attenuates bone cancer pain by inhibition of spinal astrocyte activation in a mouse model. *Anesthesiology*. 2012; 116:122–132. [PubMed: 22123524]
- Ren K, Dubner R. Neuron-glia crosstalk gets serious: role in pain hypersensitivity. *Current opinion in anaesthesiology*. 2008; 21:570–579. [PubMed: 18784481]
- Roselli CE, Resko JA. Sex differences in androgen-regulated expression of cytochrome P450 aromatase in the rat brain. *The Journal of steroid biochemistry and molecular biology*. 1997; 61:365–374. [PubMed: 9365212]
- Ruiz-Medina J, Baulies A, Bura SA, Valverde O. Paclitaxel-induced neuropathic pain is age dependent and devolves on glial response. *European journal of pain*. 2013; 17:75–85. [PubMed: 22623135]
- Schwei MJ, Honore P, Rogers SD, Salak-Johnson JL, Finke MP, Ramnaraine ML, Clohisy DR, Mantyh PW. Neurochemical and cellular reorganization of the spinal cord in a murine model of bone cancer pain. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 1999; 19:10886–10897. [PubMed: 10594070]
- Shi Y, Gelman BB, Lisinicchia JG, Tang SJ. Chronic-pain-associated astrocytic reaction in the spinal cord dorsal horn of human immunodeficiency virus-infected patients. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 2012; 32:10833–10840. [PubMed: 22875918]
- Simpson ER, Davis SR. Minireview: aromatase and the regulation of estrogen biosynthesis--some new perspectives. *Endocrinology*. 2001; 142:4589–4594. [PubMed: 11606422]
- Smeester BA, Al-Gizawiy M, Beitz AJ. Effects of different electroacupuncture scheduling regimens on murine bone tumor-induced hyperalgesia: sex differences and role of inflammation. *Evidence-based complementary and alternative medicine : eCAM*. 2012; 2012:671386. [PubMed: 23320035]
- Smeester BA, Al-Gizawiy M, O'Brien EE, Ericson ME, Triemstra JL, Beitz AJ. The effect of electroacupuncture on osteosarcoma tumor growth and metastasis: analysis of different treatment regimens. *Evidence-based complementary and alternative medicine : eCAM*. 2013; 2013:387169. [PubMed: 24228059]
- Smeester BA, Lunzer MM, Akgun E, Beitz AJ, Portoghese PS. Targeting putative mu opioid/metabotropic glutamate receptor-5 heteromers produces potent antinociception in a chronic murine bone cancer model. *European journal of pharmacology*. 2014; 743:48–52. [PubMed: 25239072]
- Wacnik PW, Eikmeier LJ, Ruggles TR, Ramnaraine ML, Walcheck BK, Beitz AJ, Wilcox GL. Functional interactions between tumor and peripheral nerve: morphology, algogen identification, and behavioral characterization of a new murine model of cancer pain. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 2001; 21:9355–9366. [PubMed: 11717369]

- Wang XW, Li TT, Zhao J, Mao-Ying QL, Zhang H, Hu S, Li Q, Mi WL, Wu GC, Zhang YQ, Wang YQ. Extracellular signal-regulated kinase activation in spinal astrocytes and microglia contributes to cancer-induced bone pain in rats. *Neuroscience*. 2012; 217:172–181. [PubMed: 22564552]
- Xu J, Zhu MD, Zhang X, Tian H, Zhang JH, Wu XB, Gao YJ. NFkappaB-mediated CXCL1 production in spinal cord astrocytes contributes to the maintenance of bone cancer pain in mice. *Journal of neuroinflammation*. 2014; 11:38. [PubMed: 24580964]
- Yao M, Chang XY, Chu YX, Yang JP, Wang LN, Cao HQ, Liu MJ, Xu QN. Antiallodynic effects of propentofylline Elicited by interrupting spinal glial function in a rat model of bone cancer pain. *Journal of neuroscience research*. 2011; 89:1877–1886. [PubMed: 21812015]
- Zhang RX, Liu B, Wang L, Ren K, Qiao JT, Berman BM, Lao L. Spinal glial activation in a new rat model of bone cancer pain produced by prostate cancer cell inoculation of the tibia. *Pain*. 2005; 118:125–136. [PubMed: 16154703]
- Zhang Y, Lu N, Zhao ZQ, Zhang YQ. Involvement of estrogen in rapid pain modulation in the rat spinal cord. *Neurochemical research*. 2012; 37:2697–2705. [PubMed: 22903468]
- Zhong YQ, Li KC, Zhang X. Potentiation of excitatory transmission in substantia gelatinosa neurons of rat spinal cord by inhibition of estrogen receptor alpha. *Molecular pain*. 2010; 6:92. [PubMed: 21143988]

Highlights

- Tumor nociception is associated with increases in spinal astrocyte activity.
- Tumor nociception is associated with increases in spinal aromatase expression.
- Administration of an aromatase inhibitor reduces tumor-induced hyperalgesia.

Fig. 1. Murine spinal cord tissue expresses aromatase

Representative western blot showing aromatase antibody specificity in naïve animals. A band corresponding to the expected molecular weight of aromatase was detected at 55 kDa. Lane labels as indicated (1 = positive control cerebellum; 2 = negative control MD-MDA 231 cells; 3-5 = naïve mice; MW = molecular weight marker).

Fig. 2. Aromatase is co-localized in astrocytes, but not neurons

Representative immunohistochemical staining of glial fibrillary protein GFAP (A), neuronal marker NeuN (B) and colocalization of aromatase in astrocytes only (A; merged) in fibrosarcoma-bearing mice. Individual images were taken at 200X; merged at 400X; scale bars = 100 μ m. White arrows indicate positive stained cell types, black arrows indicate co-localized aromatase in GFAP-positive astrocytes.

Fig. 3. Painful fibrosarcoma induces mechanical hyperalgesia

Mechanical hyperalgesia was evident in fibrosarcoma mice as early as post-implantation day 7 (PID 7) and continued through all time points tested as compared to saline-injected control mice (open squares *v.* circles; **** $p < 0.0001$). Conversely, implantation of melanoma cells into the calcaneus did not elicit changes in mechanical hyperalgesia at any of the time points tested when compared with saline controls (black squares *v.* circles; $p > 0.05$). Percent response shown as mean $\pm$ SEM, $n = 8/\text{group}$.

Fig. 4. Fibrosarcoma induces cold hypersensitivity

The numbers of spontaneous behaviors were recorded at baseline (BL) prior to injection of tumor cells or saline into the calcaneus and then following implantation of fibrosarcoma (A) or melanoma (B) cells at 7, 10 and 14 days post-injection. Changes in the number of spontaneous behaviors were observed in fibrosarcoma-bearing animals when exposed to a cold plate for 2 min at 4°C at all time points tested as compared to saline controls (A; $p < ****0.0001$). No changes were observed in melanoma-bearing mice when compared to their controls (B; $p > 0.05$). Data shown as mean number of behaviors $\pm$ SEM, $n = 5-8$ /group.

Fig. 5. Representative photomicrographs of fluorescent immunoreactivity (-ir) in B6C3 murine spinal cord
B6C3 GFAP-ir and aromatase-ir in saline control (A and C, respectively) and melanoma-tumor (B and D, respectively) mice are depicted. Representative fibrosarcoma images can be seen in Fig. 2. GFAP-ir images were taken at 200X, aromatase images at 100X; scale bar in all photos represents 100 μ m. White arrows in each image indicate positive immunostaining. Further image analysis and graphical representation was performed and is shown in Figs. 6 and 7.

Fig. 6. Astrocytes are activated in fibrosarcoma-bearing mice

Lower lumbar (L4-L5, laminae I-II, III-IV, V-VI, X) astrocyte expression (ir, immunoreactivity) was quantified in fibrosarcoma and melanoma-bearing animals at post-implantation day 14. Increased GFAP-ir in fibrosarcoma-bearing animals was observed in all laminae analyzed as compared to control (A) while no differences in GFAP-ir were seen in any laminae quantified in melanoma-bearing mice as compared to their control (B). All data shown as mean GFAP-ir $\pm$ SEM; * $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; $n = 4/\text{group}$ - 40 sections/group.

Fig. 7. The fold change in fibrosarcoma-induced aromatase activation is greater than melanoma-induced aromatase activation

All immunopositive aromatase cells were counted and expressed as a total count in fibrosarcoma (A) and melanoma (B) -bearing animals in laminae I-X of the dorsal horn in the lumbar region. Tumor-induced increases in aromatase-ir cell counts were observed in both tumor types as compared to individual controls (A-B; **** $p < 0.0001$). Fibrosarcoma animals had a significantly larger fold change in aromatase-ir when compared to melanoma-bearing animals (C). Aromatase-ir cells were examined individually in laminae I-II, III-IV, V-VI and X in fibrosarcoma-bearing mice (D). Fibrosarcoma animals had increased aromatase positive cell counts in all laminae. Data shown as mean $\pm$ SEM; * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$; $n = 4/\text{group}$ - 40 sections/group.

Fig. 8. Letrozole inhibits mechanical hyperalgesia and aromatase expression

Letrozole was given daily (10 μ g in 0.1 mL 0.3% HPC) beginning post-implantation (PID) 7 for a total of 7 days; representative images of GFAP-ir in vehicle control (A; 0.3% HPC) and letrozole-treated animals (B). Although not quantitated, GFAP-ir appeared to be reduced in letrozole-treated animals (Fig 8A v. B). Mechanical von Frey scores (C) and aromatase expression in laminae I-II, III-IV, V-VI and X (D) were evaluated on post-implantation day 14 (PID 14). Letrozole significantly reduced tumor-induced mechanical hyperalgesia and aromatase positive cell counts in laminae I-II on PID 14 as compared to controls. Data shown as mean $\pm$ SEM; * p < 0.05, **** p < 0.0001; n = 6-8/group.