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Award Number:

W81XWH-10-1-0349

TITLE:

Mechanisms of Aromatase Inhibitor-induced Musculoskeletal Symptoms

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REPORT DATE:

July 2012

TYPE OF REPORT:

Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE 01 Jul 20127		2. REPORT TYPE Annual Summary		3. DATES COVERED 01-07-2011 - 31-06-2012	
4. TITLE AND SUBTITLE Mechanisms of Aromatase Inhibitor-induced Musculoskeletal Symptoms				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-10-1-0349	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Jason Robarge E-Mail: jrobarge@iupui.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Indiana University Indianapolis, IN 46202				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT The use of aromatase inhibitor (AI) therapies in breast cancer is limited in many patients because they induce musculoskeletal pain; however, the effects of pharmacologic inhibition of estrogen synthesis on nociceptive changes in animal models of pain are unknown. To address this issue, we determined whether administration of the AI letrozole to male rats would alter nociceptive responses to mechanical or thermal stimuli. Daily dosing of 5 mg/kg letrozole in male rats induced mechanical, but not thermal hypernociception. Additionally, we have demonstrated that treatment with the aromatase inhibitors letrozole and exemstane enhances nociceptive response to ATP, suggesting sensitization to ATP may play a role in AI-induced pain. We further demonstrate that aromatase expression in rat dorsal root ganglia is maintained when these cells are cultured. In DRG cultures, letrozole treatment enhances ATP-mediated sensitization, as measured by enhanced capsaicin-evoked release of iCGRP. Further studies are needed to determine whether AIs directly alter sensory neuron function in vivo.					
15. SUBJECT TERMS aromatase, aromatase inhibitors, pain					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
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INTRODUCTION

Aromatase inhibitors (AIs) are the current standard of care for endocrine treatment of breast cancer in post-menopausal women. Yet the use of AIs is limited in many patients because they induce severe musculoskeletal pain. AI-induced pain is often intolerable and unresponsive to typical analgesics, causing many patients to discontinue therapy. By potently inhibiting the aromatase enzyme, AIs deplete plasma estrogens in humans to below post-menopausal concentrations. Although AI-induced pain is thought to be a consequence of aromatase inhibition and estrogen deprivation, alleviating symptoms with estrogenic treatments cannot be ethically tested in the clinical setting. Thus, alternative methods are needed to establish the pathophysiology and mechanisms linking aromatase inhibition to musculoskeletal pain. The *objective* of this proposal is to establish a model for AI-induced pain and investigate the potential contribution of nociceptive sensory neurons. The *central hypothesis* is that aromatase inhibition in nociceptors causes sensitization, resulting in pain during AI treatment. This project is *innovative* because it tests a novel mechanism for AI-induced pain. We anticipate that results of these studies will provide primary insight into the role of sensory neurons in pain during AI treatment.

BODY

This past year's research accomplishments for each specific task outlined in the Statement of Work are listed below:

Task 1: Determine if AI-induced hypernociception is estrogen dependent.

To address the question of whether AIs alter nociceptive thresholds in rats, we have measured withdrawal reflexes to mechanical and thermal stimulation of sensory neuron terminals in the hindpaw skin. Using the ovariectomized female rat (OVX) to simulate the physiological state following menopause, we demonstrated that letrozole administration to OVX rats enhances sensitivity to mechanical stimulation of the hindpaws. Letrozole treatment selectively altered mechanosensation, as there was no change in nociceptive withdrawal latency to noxious thermal stimulation.

Clinically, in addition to their use in breast oncology in women, aromatase inhibitors are additionally being evaluated for therapeutic use in men, including male breast cancer, pediatrics, and treating gynecomastia associated with use of aromatizable anabolic steroids (1, 2). As in women, letrozole reduces serum estrogens in men in a dose-dependent manner (3). Thus, if aromatase inhibitor induced musculoskeletal pain results from reduced estrogens, AIs would be expected to also cause musculoskeletal pain in men. However, we could not identify any studies

of aromatase inhibitors in men specifically focused on evaluating musculoskeletal symptoms. Therefore, whether AIs similarly induce musculoskeletal pain in men is unknown and determining whether AIs alter nociception in males may have important clinical implications.

As with ovariectomized female rats, estradiol concentrations in plasma of male rats are lower than the sensitivity of most assays. Despite this, inhibition of estrogen synthesis by aromatase modulates male neural physiology. For example, aromatase expression in the hippocampus protects hippocampal neurons against excitotoxicity, which is thought to be a primary cause of neuronal damage during stroke (4). In the peripheral nervous system, aromatase is expressed in DRG neurons of male rats and intrathecal letrozole administration to male rats enhances neuronal apoptosis following spinal nerve injury (5). These data support the possibility that aromatase inhibition may alter sensory neuron physiology in males and females. This hypothesis is supported by our prior studies in which systemic letrozole administration enhanced sensitivity to ATP in male rats.

To address whether letrozole alters paw withdrawal threshold to stimulation with von Frey hairs (PWT) or paw withdrawal latency to noxious thermal stimulation of the hindpaws (PWL) in male rats, we adjusted letrozole dosing used in our prior experiments with OVX rats to account for the approximately 5-fold more rapid plasma clearance of the drug by males (6). Male rats were treated with 5mg/kg letrozole (L5) or vehicle (V) daily for 15 days and PWT and PWL were measured. During the treatment period, letrozole treated rats developed significantly reduced PWT compared to vehicle treated controls (Fig 1A; $P < 0.05$, two-way RM-ANOVA evaluating treatment main effect). As shown in Figure 1A, post-hoc analysis showed letrozole-treated animals had reduced PWTs five days after initiating drug administration (Day 5, V: $5.25g \pm 0.49$ vs. L5: $1.64g \pm 0.37$, $P < 0.05$, t-test); this difference persisted through 15 days of treatment (Day 15, V: $4.88g \pm 0.60$ vs. L5: $1.18g \pm 0.16$, $P < 0.05$, t-test). In contrast to reduced PWTs observed in vehicle-treated OVX females, PWT in vehicle-treated male rats did not significantly change during the treatment period ($P > 0.05$, RM-ANOVA evaluating time main effect). After discontinuing drug administration, animals remained hypernociceptive to mechanical stimulation for at least 72 hours. However, no significant difference in PWT between treatment groups was detected 7 days after stopping treatment (day 21), suggesting the effect was reversible (Figure 1A).

We additionally determined if response latency to noxious thermal stimulation was altered by daily treatment of 5 mg/kg letrozole in males, but observed PWL was unaltered in letrozole and vehicle treated controls during drug administration (Fig 1B; $P > 0.05$, two-way RM-ANOVA evaluating time and treatment main effects).

The ability of letrozole to sensitize male rats to mechanical stimuli, without altering response to noxious thermal stimulation, is consistent with our observation of letrozole-induced mechanical hypernociception in OVX female rats. However, our studies also demonstrate that OVX rats exhibit significantly reduced PWT over time if treated with saline or vehicle

[described in the 2011 Annual Report]. Therefore, to minimize changes in nociception due to ovariectomy, male rats were used in all subsequent experiments.

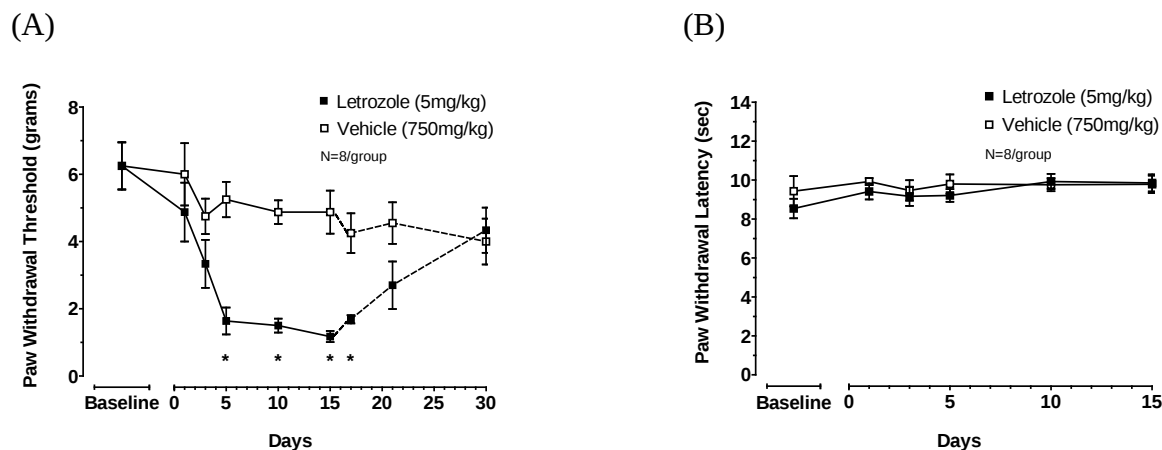


Figure 1. Daily dosing of 5mg/kg letrozole induces mechanical, but not thermal hypernociception in male rats. Each point represents the mean $\pm$ S.E.M. of (A) paw withdrawal threshold (PWT) in grams or (B) paw withdrawal latency (PWL) in seconds. Letrozole or vehicle was administered daily on days 0 through 14 (15 total doses) during which behavior was measured (solid lines). PWT continued to be measured following drug discontinuation (dashed lines). (A) After five days of treatment, PWTs of letrozole-treated rats were significantly reduced relative to vehicle-treated rats; an effect that was reversed upon drug discontinuation. An asterisk represents significant differences in PWT between treatment groups ($P < 0.05$; t-test). (B) Paw withdrawal latency to a noxious thermal stimulus was not altered during daily administration of letrozole.

Exploring enhanced ATP sensitivity as a mechanism of AI-induced hypernociception

Aromatase inhibitors augment ATP-induced overt hypernociception in male rats

Intradermal injection of purinergic receptor agonists induces dose-dependent overt nociceptive behavior in rats, as measured by hindpaw lifting and licking activity (7). Interestingly, estrogens have been shown to attenuate nociceptive responses to ATP. In response to intraplantar ATP injection, ovariectomized rats exhibit prolonged hindpaw lifting when compared to intact females, an effect partially attenuated by systemic estradiol replacement (8). *In preliminary experiments described in our 2011 Annual Report*, we confirmed these results and observed greater cumulative nociceptive response to intraplantar injection of 1000 nmol ATP in OVX versus intact female rats (intact: 9 (4, 19.5) vs. ovx: 24 (15, 35); median (25th percentile, 75th percentile); $P < 0.05$, Mann-Whitney U test).

To determine if systemic letrozole treatment facilitates overt nociceptive behavior produced by intraplantar ATP injection, male rats were treated for five days with saline (S), vehicle (V), or 5 mg/kg letrozole (L5) (i.p.). [NOTE: Preliminary results were reported in the

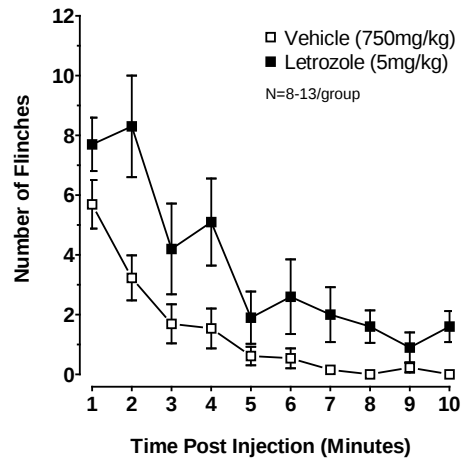
2011 Annual Report and subsequent experiments have confirmed these findings. The aggregate results are discussed here.] Twenty four hours following the last injection, flinching behavior in response to PBS or 1000 nmol ATP injections in the left hind paw was observed for 10 minutes. In all treatment groups, flinching behavior was rapidly induced following ATP administration and declined rapidly during the observation period. Interestingly, 9 of 10 letrozole-treated rats (90%) displayed nociceptive behavior from 8 to 10 minutes, whereas 7 flinches were observed in the 21 rats systemically treated with saline or vehicle (33.3%). This suggests letrozole treatment not only augments, but also slightly prolongs the nociceptive behavior in response to ATP at this dose (Fig 2A). In contrast, intraplantar PBS injections produced a small number of flinches, all of which were observed within six minutes following PBS administration, suggesting a negligible effect of restraint or injection (data not shown). In addition, systemic letrozole treatment did not alter nociceptive behavior in response to PBS relative to systemic vehicle controls (S: 1 (0, 4.5) vs. V: 1 (0, 3) vs. L: 2 (0, 3); median (25th percentile, 75th percentile); $P < 0.05$, Mann-Whitney U test). Relative to PBS injections, significant nociceptive behavior in response to 1000 nmol ATP was observed in all treatment groups ($P < 0.05$, Mann-Whitney U test). No difference in response to 1000 nmol ATP was observed between rats treated systemically with vehicle or saline (data not shown). However, systemic letrozole treatment augmented the cumulative response to ATP relative animals treated systemically with the vehicle (Fig 2B; S: 17 (9, 24) vs. V: 14 (6.5, 22) vs. L: 35 (22.25, 49.5); median (25th percentile, 75th percentile); $P < 0.05$, Mann-Whitney U test).

To determine whether letrozole enhancement of ATP-induced nociceptive behavior is drug-specific or could be a generalized effect of AIs, we evaluated overt nociceptive behavior to intraplantar ATP following systemic treatment with exemestane, a mechanism-based inhibitor of aromatase (9). Previous in vivo characterization of exemestane demonstrated dose-dependent reduction of ovarian aromatase activity in pregnant mare serum gonadotropin-stimulated adult female rats 24 hours following single doses of exemestane (s.c.), with 81% inhibition at 10 mg/kg (9). When dosed twice a day for 4 weeks (dosing s.c. 6 days/week), 10 – 100 mg/kg/day exemestane maximally inhibited ovarian aromatase activity in situ from rats with 7,12-dimethylbenzanthracene (DMBA)-induced mammary carcinoma (10). To address our question, male rats were administered vehicle (V) or 30 mg/kg exemestane (E30) for five days (i.p.), a treatment paradigm expected to produce substantial aromatase inhibition. Twenty-four hours following the last injection, we measured nociceptive behavior in response to an intraplantar injection of 1000 nmol ATP. As observed in letrozole treated animals, flinching behavior in response to ATP persisted in the last two minutes of observation in 6 of 7 exemestane-treated rats (85.7%), in contrast to 1 of 9 vehicle-treated animals (11.1%), suggesting aromatase inhibition prolongs ATP-induced nociception (Fig 2C). Intraplantar ATP induced flinching behavior in all rats, however cumulative nociceptive response to 1000 nmol ATP were significantly greater in exemestane-treated rats (Fig 2D; V: 19 (10.5, 24.5) vs. E30: 32 (18, 35); median (25th percentile, 75th percentile); $P < 0.05$, Mann-Whitney U test).

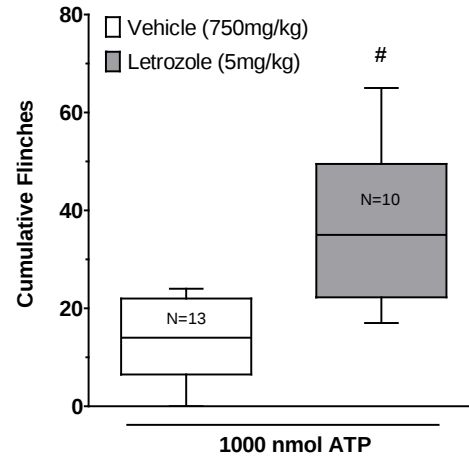
While our experiments demonstrate chronic dosing of aromatase inhibitors alters nociceptive response to intraplantar ATP, the time course in which sensitivity to ATP is enhanced is unknown. In fact, changes in nociception have been shown to develop within minutes after aromatase inhibitor treatment. For instance, experiments in which vorozole was administered intrathecally to male quail demonstrated vorozole-induced analgesia to thermal stimulation in 54°C water bath after 1 and 5 min (11). The authors did not report whether or not aromatase inhibitors alter response to tactile mechanical stimulation or inflammatory mediators such as ATP. Additionally, nociceptive flinching in response to intraplantar injection of 0.5% formalin was enhanced by 5mg/kg letrozole treatment only 15 minutes after systemic injection of the aromatase inhibitor (12). These studies support the ability of aromatase inhibition to alter nociception in an acute time frame.

Our prior pharmacokinetics study in OVX rats demonstrated letrozole distributes to dorsal root ganglia (DRG) and the brain by 15 minutes following a single dose of 1 mg/kg (i.p.); however, letrozole concentrations in the DRG were significantly higher at three hours than 15 minutes. Therefore, to address the question of whether ATP-mediated flinching is altered rapidly after AI treatment, we chose to evaluate nociceptive behavior in response to ATP three hours after a single i.p. injection. Male rats were treated with a single dose of vehicle (V) or 5 mg/kg letrozole (L5) (i.p.). Three hours following drug administration, flinching behavior in response to 1000 nmol ATP injections in the left hind paw was observed for 10 minutes. Flinching behavior was rapidly induced following ATP administration and declined rapidly during the observation period (Fig 3A). Systemic letrozole treatment augmented the cumulative response to ATP relative to animals treated systemically with the vehicle (Fig 3B; V: 7 (3, 8) vs. L: 18 (14, 22); median (25th percentile, 75th percentile); $P < 0.05$, Mann-Whitney U test).

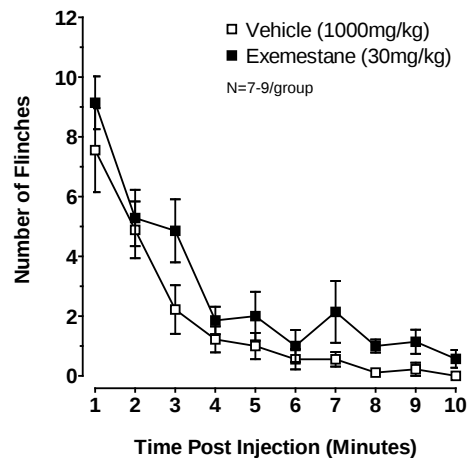
(A)



(B)



(C)



(D)

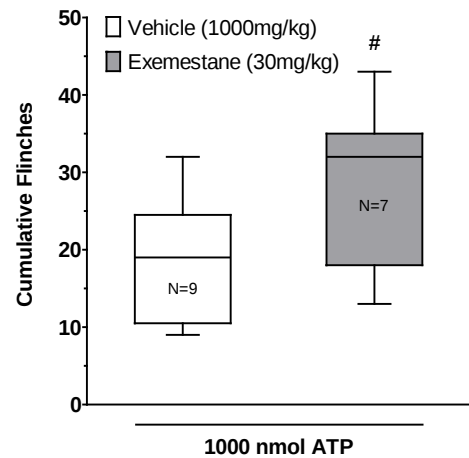
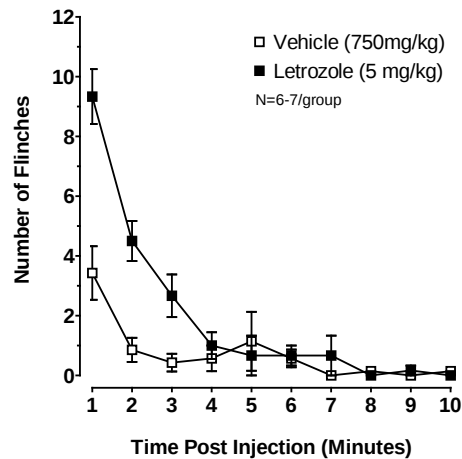


Figure 2. ATP-induced nocifensive behavior in male rats is augmented following five days of aromatase inhibitor treatment. In (A) and (C), each point represents the mean $\pm$ S.E.M. of flinches observed in one minute intervals after injection of 1000nmol ATP in rats treated for five days with letrozole, exemestane or vehicle. In (B) and (D), cumulative hind paw flinches observed in ten minutes following ATP injections are presented as boxplots for each chronic treatment group. Horizontal lines of the boxplots represent (from top to bottom): 75th percentile + (1.5 x interquartile range), 75th percentile, median, 25th percentile, 25th percentile - (1.5 x interquartile range). The pound symbol indicates significantly different ATP-induced cumulative flinches in vehicle-treated controls versus (B) letrozole and (D) exemestane-treated rats ($P < 0.05$; Mann-Whitney U test).

(A)



(B)

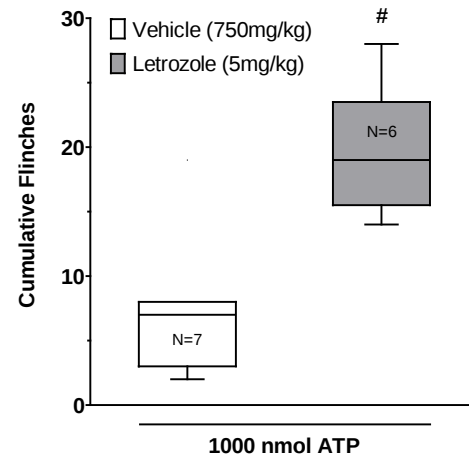


Figure 3. ATP-induced nocifensive behavior in male rats is augmented three hours after letrozole treatment. (A) Each point represents the mean $\pm$ S.E.M. of flinches observed in one minute intervals after injection of 1000nmol ATP, which was administered three hours after letrozole or vehicle treatment. (B) Cumulative hind paw flinches observed in ten minutes following ATP injections are presented as boxplots for each treatment group. Horizontal lines of the boxplots represent (from top to bottom): 75th percentile + (1.5 x interquartile range), 75th percentile, median, 25th percentile, 25th percentile - (1.5 x interquartile range). The pound symbol indicates significantly different ATP-induced cumulative flinches in rats treated with vehicle versus letrozole ($P < 0.05$; Mann-Whitney U test).

Task 2: Determine if AIs induce nociceptor sensitization.

The perception of pain is an integrated response to noxious and potentially damaging stimuli. This integrated response is initiated by specialized sensory neurons that detect and encode noxious stimuli. These neurons are collectively referred to as nociceptors. Though activation of sensory neurons is often the initiating event in the transduction of nociceptive stimuli, the perception of pain involves processing and transmission of encoded noxious stimuli by second-order sensory neurons in the spinal cord and subsequent activation of multiple brain regions. Furthermore, the integration and summation of nociceptor activity in the spinal cord determines activation of projection neurons to the brain (recognition of pain), and the activation of motor neurons (reactive reflex). In inflammatory and neuropathic pain, nociceptors can become sensitized to stimulation, such that they produce action potentials in response to normally sub-threshold stimulation.

Sensory neurons exposed to sex steroid display an altered response to a variety of inflammatory mediators, suggesting that endogenous steroids play an important role in nociception. *In vitro* studies have shown that estrogen has effects consistent with reduced activation of sensory neurons through estrogen receptor mediated signaling (13-15). For example, prolonged exposure of nociceptive neurons to 17 β -estradiol inhibits capsaicin-induced ion currents through the TRPV1 cation channel, an important chemical and thermal nociceptive transducer (15). In addition to steroids supplied by circulation in the blood, there are neurosteroids metabolized by cytochrome P450s within first and second-order sensory neurons. These neurosteroids have been proposed to rapidly cross membranes and alter neuronal excitability. Immunohistochemical staining has recently identified aromatase in sensory neurons, suggesting estrogens synthesized within sensory neurons may directly alter their physiology via intracrine or paracrine signaling (5). Although AIs have been shown to cross the blood brain barrier and also accumulate in the dorsal root ganglia [unpublished data], the impact of aromatase inhibition by AIs on sensory neurons is unknown (16).

Based on the results of our behavioral studies, we hypothesize that aromatase inhibitors directly sensitize sensory neurons or enhance sensitivity to extracellular ATP. Furthermore, one potential mechanism of this sensitizing action of AIs is through augmented release of neuropeptides from sensory neurons.

Sensory neuron cultures were prepared using a modification of our previously published protocol (17). Dorsal root ganglia of adult male rats were dissected into ice-cold PUCKS saline solution and single-cell suspensions were produced by enzymatic digestion and trituration. Approximately 30,000 cells per well were plated into 12-well Falcon culture plates coated with PDL and laminin. Cells were grown in 3% CO₂ in phenol red-free DMEM/F12 media supplemented with 3 mM glutamine, 50 units/mL penicillin, 50 μ g/mL streptomycin, 30 μ M FDU, 75 μ M uridine, 10% (v/v) heat-inactivated horse serum, and 30 ng/mL nerve growth factor.

To address this hypothesis, we first characterized aromatase expression in acutely dissociated DRGs and DRG neurons cultured for 12 days using westerns blots and antibodies directed against aromatase (mouse anti-human monoclonal Ab; Serotec) and actin (mouse anti-actin; BioRad). In protein lysates from DRGs and ovaries, we observed a single immunoreactive band at 55kDa, corresponding to the expected molecular weight of aromatase (Fig 4A). However, aromatase immunoreactivity was not detected in sensory neurons after 12 days in culture, suggesting aromatase expression may be downregulated during this culture period (Fig 4A). To address this possibility, we probed for aromatase expression at several time points after neurons were cultured. Using a high-sensitivity ECL kit, immunoreactivity bands corresponding to aromatase were observed at days 1, 4, 7, and 12 in culture (Fig 4B). This suggests that aromatase expression is maintained at low levels in culture and its expression level is relatively consistent throughout a 12-day culture. We additionally probed for two internal controls, beta-actin and Hypoxanthine-guanine phosphoribosyltransferase (HPRT). Both internal controls showed increased expression with increasing time in culture. The expression pattern of these endogenous controls may reflect the growth of glia and fibroblasts in the culture, as it is a consistent phenomenon in our experiments.

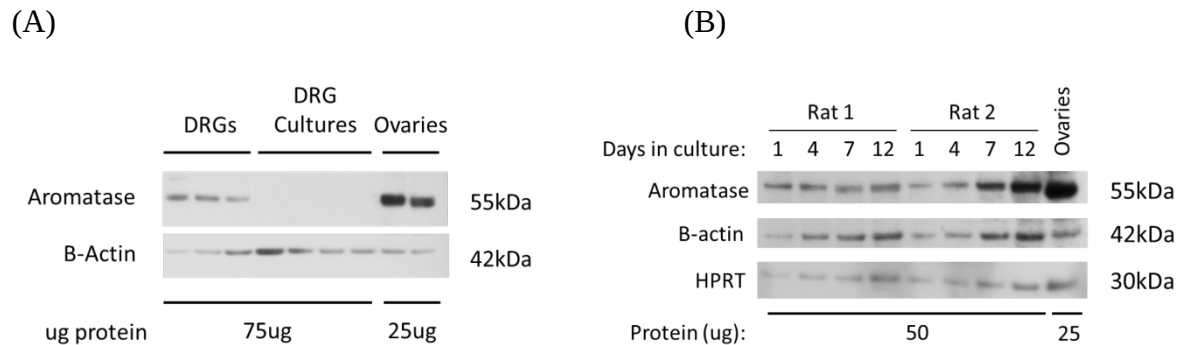


Figure 4. Aromatase is expression is maintained in cultured DRGs. (A) Aromatase immunoreactivity in: DRG lysate from three male rats, independent 12-day old male rat DRG cultures, and ovarian lysate from two female rats. Immunoreactivity detected with a high sensitivity HRP substrate following incubation with 1:2000 dilution of HRP-conjugated anti-mouse secondary antibody. (B) Aromatase immunoreactivity from independent 12-day old male rat DRG cultures. Immunoreactivity detected with SuperSignal West Femto Chemiluminescent Substrate (Thermo Fisher Scientific) following incubation with 1:100,000 dilution of HRP-conjugated anti-mouse secondary antibody.

Dorsal root ganglia are multi-cellular tissues containing cell bodies of neurons, as well as a number of non-neuronal cell types including glia, fibroblasts, and cells of the circulatory system. Therefore, because DRG cultures contain mixed cell types, the relative content of cell types within a given culture depends on culture conditions (i.e. presence of mitotic inhibitors to suppress glial growth or the presence of growth factors to promote neuronal survival). Our westerns indicate aromatase expression throughout the culture period in which functional studies

are most often conducted with this preparation (i.e. ≤ 12 days); however, we do not know whether aromatase is expressed in neurons, non-neuronal cells, or both. Therefore, aromatase expression in cultured cells was determined by immunocytochemistry (ICC). Cells plated at a density of 60,000 cells per well. After 7 days in culture, cells were fixed in 4% paraformaldehyde, washed and blocked in 10% bovine serum albumin, and then probed with antibodies against aromatase (Serotec) and the neuron-selective marker, anti-human Protein Gene Product 9.5 (PGP9.5; Serotec). Plates were stained with DAPI under a glass coverslip. Images were acquired with the use of a Nikon Ti microscope equipped with a digital CCD camera and NIS Elements image analysis software (Melville, NY). While we did not quantify immunoreactivity in these cultures, the level of aromatase immunoreactivity suggested low expression. Thus, aromatase immunoreactivity by ICC was consistent with our western blots. Our ICC data indicate that aromatase immunoreactivity in cultured adult rat sensory neurons is largely restricted to neurons, many of which were co-labeled with PGP9.5 (Fig 5). Further experiments will be conducted to identify whether aromatase expression co-localizes with functional neuronal populations, such as TRPV1 or CGRP expressing sensory neurons.

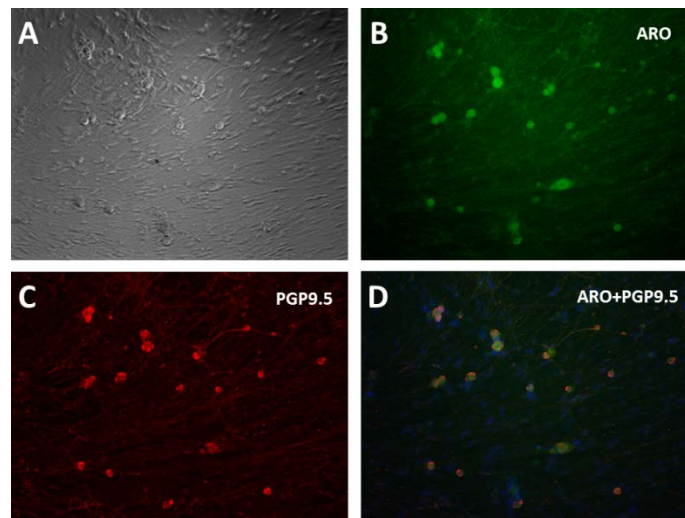


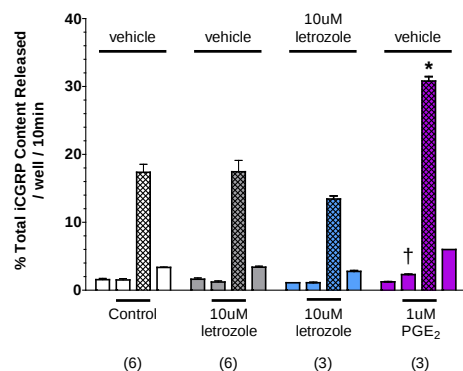
Figure 5. Aromatase and PGP9.5 immunoreactivity in cultured sensory neurons. Representative photomicrographs of double-labeling immunocytochemistry. The immunolabel is shown in the upper right hand corner of each image. (A) Bright field image of microscope field. (B) Aromatase-immunoreactive cells. (C) PGP9.5-immunoreactive neurons. (D) Neurons displaying immunoreactivity to aromatase and PGP9.5 antibodies. Co-labeled neurons appear yellow due to overlay of single-labeled images. DAPI staining of nuclei from neurons and non-neuronal cells are visible in blue.

To address whether aromatase inhibitors directly alter the basal or evoked release of neuropeptides in sensory neurons, cells were acutely and chronically treated with letrozole and subsequently basal and evoked immunoreactive calcitonin gene related peptide (iCGRP) release was measured. Specifically, iCGRP release from sensory neurons was measured by radioimmunoassay after 7 to 9 days in culture, as previously described (17). Cells were treated with vehicle or 10 μ M letrozole during the 3 days prior to measuring iCGRP release. Cells were additionally treated with vehicle or 10 μ M letrozole during the experiment. As a positive control for sensitization, we treated cells with 1 μ M prostaglandin E₂, a prostainoid previously shown by Dr. Vasko's lab to augment neuropeptide release from cultured sensory neurons evoked by the inflammatory mediator bradykinin and TRPV1-selective agonist capsaicin (17, 18). Given the sensitivity of the radioimmunoassay, iCGRP content was sufficient at these time points to reproducibly measure basal neuropeptide release. iCGRP release was measured from sequential 10-minute incubations in 400 μ L HEPES buffer maintained at 37⁰C. Basal iCGRP release was determined by incubating cells in HEPES buffer alone. Subsequently, cells were exposed to drugs diluted in HEPES to determine their effect on basal release. Then, the ability of drugs on evoked release was determined by re-exposing cells to drugs in the presence of capsaicin or 30 mM extracellular potassium (HEPES buffer with potassium chloride substituted for equimolar sodium chloride). Lastly, cells were re-exposed to HEPES buffer alone to demonstrate basal release could be re-established. To measure remaining iCGRP content, cells were lysed in 0.1 N HCl. Buffer was collected following each incubation and assayed for iCGRP.

The TRPV1-selective agonist capsaicin and high extracellular potassium were chosen for these experiments as they stimulate peptide release from different nociceptor populations expressing CGRP. TRPV1 is a non-selective cation channel that can be activated by temperatures greater than 43°C and chemical agonists such as capsaicin. TRPV1-expressing sensory neurons have been shown to contribute to development of inflammatory pain and largely innervate muscle and viscera (19, 20). However, CGRP expression is not restricted to TRPV1-containing neurons, as only 50% of adult rat sensory neurons in the DRG show co-localization of these molecules (21). CGRP is additionally expressed in roughly 30% of neurons in the DRG that bind isolectin B₄, a marker for cutaneous innervating sensory neurons (20). In contrast to capsaicin, high extracellular potassium depolarizes the plasma membrane of all CGRP-expressing sensory neurons, inducing CGRP release from neurochemically less defined sensory neurons.

As shown in Figure 6, sensory neurons iCGRP release evoked by 30nM capsaicin or high extracellular potassium (30mM) was not altered following exposure to 10 μ M letrozole. In contrast, 1 μ M PGE₂ sensitized these neurons to iCGRP release evoked by both stimuli. Also, letrozole exposure did not alter basal release of iCGRP, suggesting the drug does not directly alter excitability of sensory neurons.

(A) 30nM capsaicin



(B) 30mM KCl

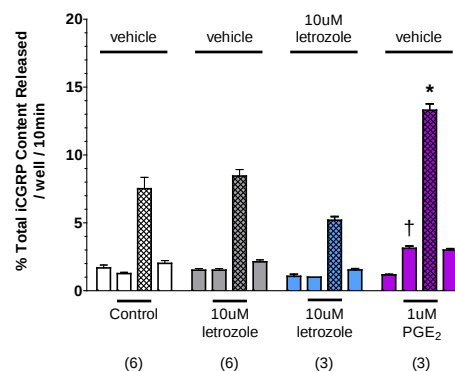
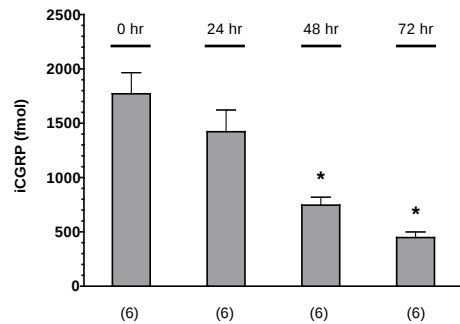


Figure 6. Letrozole does not alter basal or evoked release of iCGRP in cultured sensory neurons. The ordinates represent the mean $\pm$ S.E.M. of the amount of peptide (in femtomoles) released per well during 10 minute incubations, normalized to the total amount of peptide measured in each well (for the number of wells indicated in parentheses). Cells within each treatment group (colored) were chronically treated with vehicle or 10 μ M letrozole during the 3 days prior to measuring peptide release (indicated by lines above the columns). Cells were additionally treated during the experiment to measure iCGRP release (indicated by lines below the corresponding columns). iCGRP release was measured from four sequential incubations (indicated by columns ordered left to right), during which cells in each well were exposed to: HEPES buffer alone; buffer in the presence of the indicated treatment; (A) 30nM capsaicin or (B) high potassium buffer (30mM KCl) in the presence of the indicated treatment (shaded); HEPES buffer alone. Crosses indicate statistically significant difference in basal iCGRP release in the absence versus presence of the indicated treatment ($\dagger P < 0.05$; t-test). Asterisks indicate statistically significant difference in capsaicin or high potassium-evoked iCGRP release in the absence versus presence of the indicated treatment ($* P < 0.05$; t-test).

Sensory neuron cultures used for experiments shown in Figure 5 were grown in the presence of 10% horse serum. While letrozole did not alter evoked release of neuropeptides, it is possible that aromatase substrates (testosterone, androstenedione) or products (estradiol, estrone) formed by aromatization present in the serum masked our ability to detect an effect of letrozole. Therefore, we evaluated the effect of serum deprivation iCGRP release. Cells were grown in the absence of serum (media supplemented with 30 ng/mL NGF) for 0, 24, 48, and 72 hours prior to measuring basal iCGRP release and release evoked by 30nM capsaicin. As shown in Figure 7A, serum deprivation significantly reduced the total iCGRP content in cultured sensory neurons, with significant reductions in cells grown in serum-free culture for 48 hours. Serum deprivation also significantly altered resting release of iCGRP and augmented capsaicin-evoked iCGRP release, with no apparent time-dependent effect (Fig 7B). We then evaluated whether 10 μ M letrozole alters iCGRP release from sensory neurons grown in the absence of serum for 24 hours. While in serum free media, cells were treated with 10 μ M letrozole or vehicle and subsequently treated with these compounds or 1 μ M ATP during the iCGRP release experiment. ATP served as a positive control for sensitization of sensory neurons to capsaicin-evoked iCGRP release, as previously shown (22). Sensory neurons grown in the absence of serum for 24 hours were not sensitized by letrozole treatment; whereas neurons exposed to 1 μ M ATP displayed augmented capsaicin-evoked iCGRP release (Fig 8).

(A)



(B)

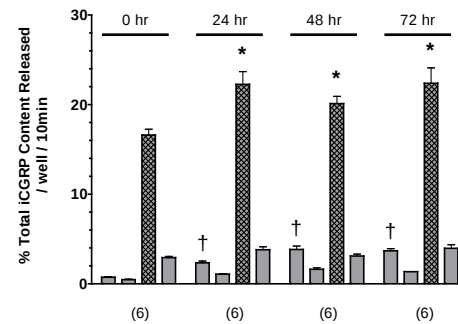


Figure 7. Serum deprivation reduces iGRP content and enhances capsaicin-evoked iGRP release. Cells were cultured for 0, 24, 48, or 72 hours in serum-free media (indicated by lines above the columns) prior to measuring capsaicin-evoked iGRP release and total iGRP content. All cells were supplemented with 30 ng/mL NGF during the last 72 hours in culture. (A) The ordinates represent the total amount of iGRP peptide (mean $\pm$ S.E.M. femtomoles) measured for the indicated number of wells (in parentheses). (B) The ordinates represent the mean $\pm$ S.E.M. of the amount of peptide (in femtomoles) released per well during 10 minute incubations, normalized to the total amount of peptide measured in each well (for the number of wells indicated in parentheses). iGRP release was measured from four sequential incubations (indicated by columns ordered left to right), during which cells in each well were exposed to: HEPES buffer; HEPES buffer alone; 30nM capsaicin (shaded); HEPES buffer. Crosses indicate statistically significant difference in basal iGRP release given the duration of serum deprivation ($\dagger P < 0.05$; t-test). Asterisks indicate statistically significant difference in capsaicin-evoked iGRP release given the duration of serum deprivation (* $P < 0.05$; t-test).

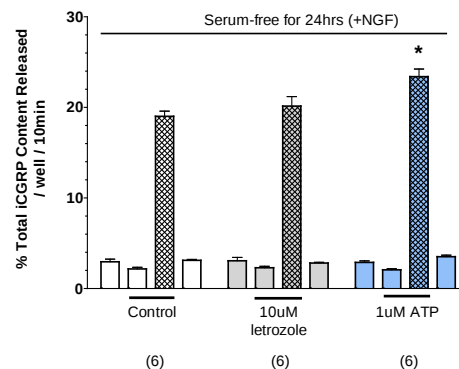


Figure 8. Following 24 hours of serum deprivation, letrozole does not alter capsaicin-evoked iGRP release or ATP-mediated sensitization. Sensory neurons were cultured in the absence of serum for 24 hours prior to measuring peptide release. All cells were supplemented with 30 ng/mL NGF during the last 24 hours in culture. Cells were additionally treated during the experiment to measure iGRP release (indicated by lines below the corresponding columns). iGRP release was measured from four sequential incubations (indicated by columns ordered left to right), during which cells in each well were exposed to: HEPES buffer alone; buffer in the presence of the indicated treatment; 30nM capsaicin in the presence of the indicated treatment (shaded); HEPES buffer alone. Crosses indicate statistically significant difference in basal iGRP release in the absence versus presence of the indicated treatment ($\dagger P < 0.05$; t-test). Asterisks indicate statistically significant difference in capsaicin-evoked iGRP release in the absence versus presence of the indicated treatment (* $P < 0.05$; t-test).

ATP is an endogenous proinflammatory mediator that excites sensory neurons by activation of P2X and P2Y purinergic receptors. When intradermally administered *in vivo*, ATP induces pain in humans and nocifensive behavior in rats (23, 24). When applied directly to sensory neurons *in vitro*, ATP induces spontaneous action potentials and enhances release of neuropeptides (22, 25). Several studies suggest estrogen may alter the effect of ATP-induced nocifensive behavior *in vivo*, and that these effects may be mediated, in part, by effects of estradiol on ATP-mediated signaling in sensory neurons (8, 13, 26). Our behavioral experiments show that systemic treatment with aromatase inhibitors augments overt hypernociceptive response to intraplantar ATP. However, whether letrozole alters the sensitivity of nociceptive neurons to ATP is unknown. To address this question, we determined whether letrozole would augment ATP-mediated sensitization in cultured sensory neurons.

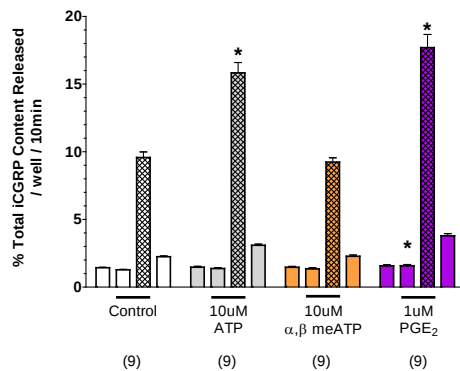
In cultured neonatal DRG neurons, Huang et al. 2003 observed significantly increased capsaicin-stimulated iCGRP release following treatment with increasing concentrations of ATP (0.1 to 300 μ M) (22). However, when the cells were treated with the P2X-selective agonist α,β meATP, they observed a significant reduction in capsaicin-evoked peptide release. Interestingly, the culture media used in these experiments contained phenol red, a known estrogen receptor agonist that was subsequently found to also non-competitively antagonize P2X channels (27). Therefore, using sensory neurons isolated from adult rats and cultured in phenol red-free culture media, we re-evaluated the effect of ATP and α,β meATP on iCGRP release evoked by capsaicin and high extracellular potassium.

As shown in Figure 9A, exposing adult sensory neurons in culture to 10 μ M ATP and 10 μ M α,β meATP did not increase the release of iCGRP from sensory neurons. As previously demonstrated by Huang et al., pre-exposing these neurons to 10 μ M ATP prior to and throughout stimulation with 30 nM capsaicin resulted in enhanced release of iCGRP relative to vehicle-treated controls. Alternatively, exposure to 10 μ M α,β meATP prior to and throughout stimulation with 30 nM capsaicin, did not alter iCGRP release relative to vehicle-treated cells. Therefore, the presence of phenol red does not appear to be a significant confounder in the effects of α,β meATP on release of neuropeptides from sensory neurons. In contrast to the ability of ATP to sensitize capsaicin-evoked neuropeptide release, neither 10 μ M ATP or 10 μ M α,β meATP altered iCGRP release following depolarization of the cell membranes induced by 30 mM extracellular potassium (Fig 9B). However, 1 μ M PGE2 significantly augmented release evoked by high extracellular potassium.

Finally, to address whether acute letrozole treatment alters the ability of ATP to augment stimulated release of neuropeptides, sensory neurons were treated with vehicle, 10 μ M letrozole, 1 μ M ATP, and the combination prior to and throughout stimulation with 10 nM capsaicin. Lower ATP and capsaicin concentrations were chosen to reduce the probability that neurons are maximally sensitized by ATP following stimulation with capsaicin. Exposing sensory neurons to 1 μ M ATP and 10 μ M letrozole did not alter basal iCGRP release relative to controls (Fig 10). As expected, exposure to 1 μ M ATP prior to and throughout stimulation with 10 nM capsaicin

increased neuropeptide release relative to controls (Fig 10). While neurons exposed to 10 μ M letrozole did not increase capsaicin-induced release of iCGRP, co-treatment with 10 μ M letrozole and 1 μ M ATP prior to and throughout stimulation with 10nM capsaicin increased neuropeptide release relative to cells treated with 1 μ M ATP (Fig 10).

(A) 30nM capsaicin



(B) 30mM KCl

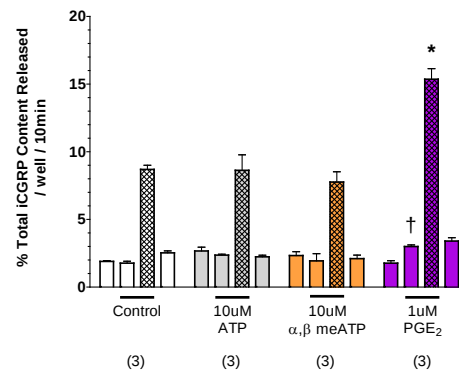


Figure 9. ATP selectively enhances capsaicin-evoked iCGRP release. The ordinates represent the mean $\pm$ S.E.M. of the amount of peptide (in femtomoles) released per well during 10 minute incubations, normalized to the total amount of peptide measured in each well. The numbers of wells analyzed per condition are given in parentheses. Within each treatment group (colored), individual columns represent sequential incubations (ordered left to right) during which cells in each well were exposed to: HEPES buffer; buffer in the presence of the indicated treatment; (A) 30nM capsaicin or (B) high potassium buffer (30mM KCl) in the presence of the indicated treatment (shaded); HEPES buffer. Crosses indicate statistically significant difference in basal iCGRP release in the presence of the indicated treatment ($\dagger P < 0.05$; t-test). Asterisks indicate statistically significant difference in iCGRP release caused by capsaicin or high potassium in the presence of the indicated treatment ($* P < 0.05$; t-test).

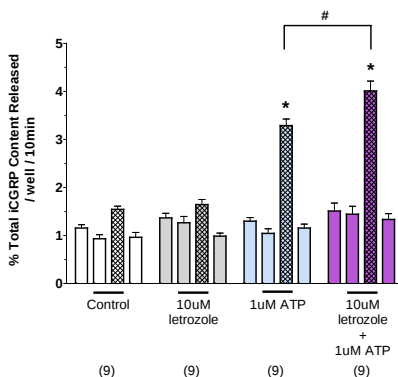


Figure 10. Letrozole augments ATP-induced enhancement of iCGRP release evoked by 10nM capsaicin. The ordinates represent the mean $\pm$ S.E.M. of the amount of peptide (in femtomoles) released per well during 10 minute incubations, normalized to the total amount of peptide measured in each well. The numbers of wells analyzed per condition are given in parentheses. Within each treatment group (colored), individual columns represent sequential incubations (ordered left to right) during which cells in each well were exposed to: HEPES buffer; buffer in the presence of the indicated treatment; (A) 10nM capsaicin in the presence of the indicated treatment (shaded); HEPES buffer. Asterisks indicate significant difference in capsaicin-evoked iCGRP release in the presence of the indicated treatment versus controls ($P < 0.05$; t-test). The pound symbol indicates a significant difference in capsaicin-evoked iCGRP release in the presence of ATP versus ATP and letrozole ($P < 0.05$; t-test).

While the mechanism by which letrozole can alter sensitivity of sensory neurons to ATP is unknown, these data are consistent with our behavioral data and support that possibility that the interaction between aromatase inhibition and ATP-mediated signaling at the level of the sensory neuron represent a component of AI-induced hypernociception. Additionally, the potential mechanistic underpinnings identified in these experiments provide direction, and novel hypotheses to address evaluating changes in capsaicin-evoked neuropeptide release in spinal cord slices.

Conduction of action potentials following sufficient stimulation of sensory neurons *in vivo* results in release of neurotransmitters from sensory neuron central terminals in the dorsal horn of the spinal cord. Modulation of neurotransmission at this level may determine how signals from primary afferent nociceptors are integrated into higher centers of the nervous system and subsequently interpreted as pain. We propose that aromatase inhibition facilitates the release of neuropeptides from nociceptor central terminals within the dorsal horn of the spinal cord. To address this hypothesis, AI-induced changes in nociceptor activity will be determined by measuring basal and evoked iCGRP release from rat spinal cord slices using a radioimmunoassay. The degree of nociceptor sensitization will be measured by a change in iCGRP release after activating TRPV1-expressing nociceptor central terminals with capsaicin. It has been shown that inducing inflammation *in vivo* or perfusing inflammatory mediators in rat spinal cord slices results in augmentation of capsaicin-evoked iCGRP release (28-30). While we have shown ATP sensitizes isolated sensory neurons to capsaicin-evoked iCGRP release and

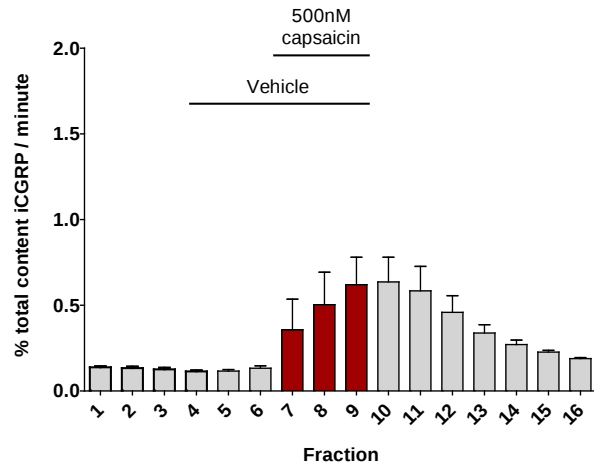
intrathecal injection of ATP-analogs induces mechanical hypernociception, it is not known whether ATP sensitizes basal or evoked release of neuropeptides from the central terminals of sensory neurons (22, 31). Due to the nature of the spinal cord release experiment, tissue from each animal can be used to measure capsaicin-evoked release, as well as the effect of an additional treatment, such as ATP (each animal serves as its own control). In this manner, we can test the effect of systemic AI versus vehicle treatments on iCGRP release given both spinal cord treatments. I have conducted preliminary experiments evaluating the effect of ATP on capsaicin-evoked neuropeptide release from the central projections of sensory neurons.

Detailed methods to measure iCGRP release from rat spinal cord slices have been described elsewhere (30, 32). Briefly, rats were anesthetized with CO₂, sacrificed by decapitation, and the spinal cord was extruded from the spinal column using ice-cold HEPES buffer. After removing the dura, a 1 cm section of the lumbar enlargement was isolated with two transverse cuts and then bisected into two halves, each containing the dorsal and ventral horns. Tissue pieces were spatially separated on a Teflon disc and diced into 0.3 mm x 0.3 mm cubes using the McIlwain tissue chopper. Chopped tissue from each spinal cord half was placed in separate perfusion chambers. Tissues were perfused at 0.5 mL/min with HEPES buffer aerated with 95% O₂ / 5% CO₂ maintained at 37°C. To stabilize neuropeptide release, tissues were perfused for 20 minutes prior to collecting perfusate. Perfusate was collected in 3-minute intervals (1.5mL per fraction). Basal release was measured by perfusing tissues with HEPES buffer in the absence of drugs for 9 minutes. To test the effect of drugs on basal release, tissues were perfused with HEPES buffer in the presence or absence of drugs for 9 minutes. Next, to test the effect of drugs on capsaicin-evoked release, tissues were perfused with HEPES buffer containing 250 nM or 500 nM capsaicin in the presence or absence of drugs for 9 minutes. Finally, to re-establish basal neuropeptide release, tissues were perfused with HEPES buffer alone for 21 minutes. Residual tissue was collected to measure the remaining iCGRP peptide content. iCGRP was measured from perfusates and totals using a radioimmunoassay.

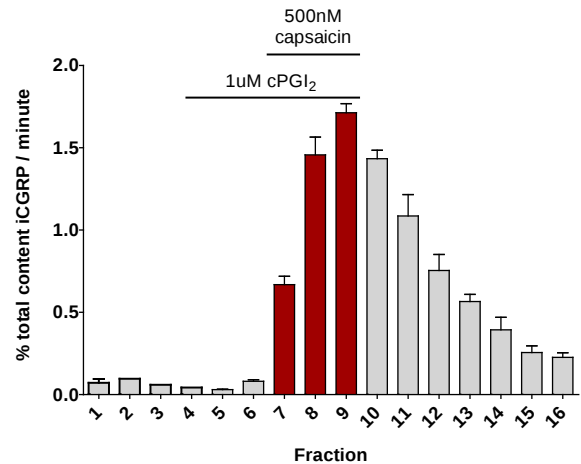
In preliminary experiments, I chose to establish iCGRP release evoked by 500 nM capsaicin in naïve male rats and demonstrate sensitization by a prostaglandin previously shown to augment the release of CGRP from sensory neuron central terminals (positive control for future experiments) (28). Tissue from one half of each rat spinal cord was perfused with 1 µM carbaprostacyclin (cPGI₂), whereas tissue from the other half was perfused with vehicle. Tissues from both halves were stimulated with 500 nM capsaicin to determine whether 1 µM carbaprostacyclin would augment capsaicin-evoked iCGRP release, as previously described. As shown in figure 11, carbaprostacyclin augmented capsaicin-evoked iCGRP release from spinal cord tissue. In additional preliminary experiments, we examined whether iCGRP release evoked by 250 nM capsaicin would be augmented by 10 µM ATP, a concentration that significantly augments capsaicin-stimulated iCGRP release in our sensory neuron cultures. At this ATP concentration, we did not observe an effect of ATP on capsaicin-evoked release (Fig 11C). In future experiments, we will evaluate the effect of higher ATP concentrations on neuropeptide

release from spinal cord slices. If ATP is shown to have no effect in this experiment, we will proceed with determining whether systemic letrozole treatment alters iCGRP from sensory neuron central terminals, as measured by changes in iCGRP release from spinal cord slices.

(A)



(B)



(C)

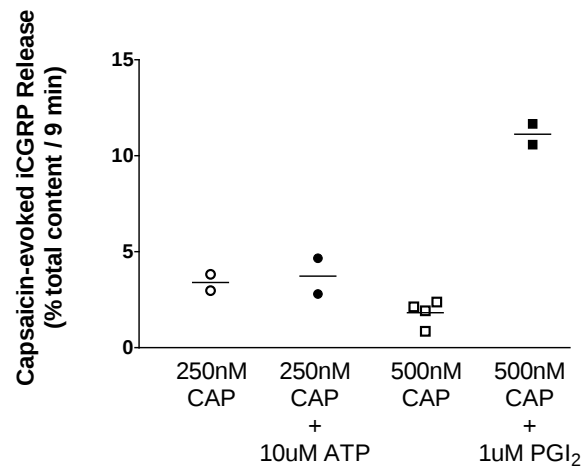


Figure 11. Effect of ATP and PGI₂ on capsaicin-evoked iCGRP release from spinal cord slices. (A) and (B). Each column represents the mean $\pm$ S.E.M. of basal release (grey columns) or capsaicin-evoked release (red columns) of iCGRP released per during 3 minutes of tissue perfusion, normalized to the total amount of peptide measured in each tissue. (A) Tissue was treated with vehicle for 9 minutes prior to and throughout stimulation with 500 nM capsaicin. (B) Tissue was treated with 1 μ M carbaprostacyclin for 9 minutes prior to and throughout stimulation with 500 nM capsaicin. (C) Evoked iCGRP release was calculated by subtracting release measured during fractions 4-6 from the evoked release during fractions 7-9. The symbols represent evoked release measured from individual tissue samples.

Breast Cancer Training Program

As outlined in the proposal for this award, a key component of this Predoctoral award was a training program that will facilitate my career in translational breast cancer research. To this end, I have been an active participant in both the Department of Pharmacology and Toxicology and the Division of Clinical Pharmacology within the School of Medicine. A number of aspects of the training program were outlined in my proposal for this award and I will briefly review my activities here:

I am a Ph.D. candidate in my department in good standing, have met the requirements for committee meetings, and currently on schedule to graduate in the spring of 2013. I attend weekly data meetings from Dr. Flockhart's and Dr. Vasko's laboratories. I also attend and have presented at semi-regular conference calls among COBRA (Consortium on Breast Cancer Pharmacogenomics) investigators, which includes several clinical oncologists. I am an active participant in the weekly Division of Clinical Pharmacology journal club and have presented several research articles to this group. I also participate in the student run Department of Pharmacology and Toxicology journal club. I have presented my research in several forums during the last year: in the Department of Pharmacology and Toxicology Seminar, in the Division of Clinical Pharmacology Seminar, and additionally at a weekly meeting of sensory neuron biologists and the IU Stark Neuroscience Research Institute. In addition to a poster presentation at the Era of Hope meeting in 2011 (Orlando, FL), I also presented my research at the Society for Neuroscience Annual Meeting in 2011 (Washington, D.C.). In addition, Pam Haschke, a breast cancer advocate supporting this research proposal, has been sent research updates. In summary, I have been continually engaged in my proposed training program and have made progress in the outlined activities.

KEY RESEARCH ACCOMPLISHMENTS

For the task outlined in the statement of work designed to determine if AI-induced hypernociception is estrogen dependent, the key research accomplishments are:

1. Confirmed that letrozole treated rats exhibit increased response to the algogen ATP, suggesting a possible mechanism by which letrozole increases pain.
2. Demonstrated that exemestane treatment also augments nociceptive response to ATP, suggesting enhanced sensitivity to ATP is mediated by aromatase inhibition.
3. Demonstrated that ATP-induced nocifensive behavior is augmented only three hours after letrozole treatment, suggesting AIs may rapidly alter sensitivity to an experimentally administered algogen.

For the task outlined in the statement of work designed to determine if AIs induce nociceptor sensitization, the key research accomplishments are:

1. Demonstrated that aromatase is expressed in DRGs and its expression is maintained in DRG cultures. Additionally showed aromatase is expressed in neurons in DRG cultures.
2. Demonstrated that letrozole does not directly alter basal iCGRP release, and additionally does not alter capsaicin or high potassium-induced iCGRP release in sensory neuron cultures.
3. Demonstrated that ATP augments iCGRP release evoked by capsaicin, but not high extracellular potassium.
4. Established that letrozole augments ATP-induced enhancement of iCGRP release.

REPORTABLE OUTCOMES

Abstracts

1. Jason D. Robarge, Djane B. Duarte, Todd C. Skaar, Michael R. Vasko, David A. Flockhart. The aromatase inhibitor letrozole produces mechanical hypernociception in the rat. For DOD BCRP Era Of Hope 2011.
2. Jason D. Robarge, Djane B. Duarte, Todd C. Skaar, Michael R. Vasko, David A. Flockhart. The aromatase inhibitor letrozole produces mechanical hypernociception in the rat. For Society for Neuroscience Meeting 2011.

Papers in preparation

1. *Running Title*: Aromatase inhibitors induce mechanical hypernociception and enhance ATP-induced hyperalgesia *in-vivo*.

Authors (no particular order): Jason D. Robarge M.S.¹, Djane B. Duarte Ph.D.¹, Todd C. Skaar Ph.D.^{1,2}, David A. Flockhart M.D. Ph.D.^{1,2}, Michael R. Vasko Ph.D.¹

¹Department of Pharmacology and Toxicology, ²Department of Medicine, Division of Clinical Pharmacology, Indiana University School of Medicine, Indianapolis, IN

Journal Preference: Pain

Presentations

1. “Developing an Experimental Model of Aromatase Inhibitor-induced Musculoskeletal Pain”, IU Stark Neuroscience Institute, Sensory Neuron Group Meeting, September 2011
2. “An experimental model of aromatase inhibitor-induced pain: The dark side of breast cancer endocrine therapy”, IU School of Medicine, Department of Pharmacology and Toxicology Seminar, October 2011
3. “Losing estrogen is such a pain!: Towards an experimental model of aromatase inhibitor-induced musculoskeletal syndrome”, IU School of Medicine, Division of Clinical Pharmacology Seminar, March 2012

CONCLUSION

Our behavioral experiments have revealed two major findings. First, letrozole induces mechanical hypernociception in both OVX and male rats. Second, male rats treated with letrozole and exemestane have enhanced sensitivity to the algogen ATP. AI-mediated enhancement of sensitivity to ATP occurs within hours of systemic drug administration. In contrast, letrozole-induced reduction in PWT to von Frey hair stimulation is significant five days after initiating drug treatment. However, the ability of letrozole to acutely sensitize ATP-induced nociceptive behavior is consistent with the rapid distribution of the drug to tissues that mediate nociceptive processing [*data presented in the 2011 Annual Report*]. Whether these observations are mechanistically related is unclear at this time and further experiments are needed to address whether altered ATP release or changes in purinergic signaling pathways are responsible for AI-induced mechanical hypernociception.

Interestingly, *as described in our 2011 Annual Report*, systemic replacement of estradiol did not prevent letrozole-induced hypernociception in OVX rats. The estradiol concentration of the implant chosen has been shown to maintain plasma estradiol concentrations of 8 to 9 pg/mL, near or slightly higher than that described for OVX and male rats. Plasma estrogen concentrations are a product of the net production of estrogen in all tissues and the tissue-specific concentration gradient with plasma. Therefore, as has been described in humans, plasma estrogen levels in rats may not correlate with tissue estrogen levels [Lonning JSMB 2009]. Thus, the plasma estradiol concentrations produced in our add-back experiment may not have resulted in sufficient tissue concentrations to inhibit the effects of letrozole. One experimental approach to address this problem is to increase plasma estradiol concentrations by supplementing additional estradiol in the implanted pellets. However, estradiol is known to augment nociceptive responses to other inflammatory mediators such as bradykinin [Rowan J Pharmacol Exp Ther. 2010]. Therefore, pro-nociceptive versus anti-nociceptive effects of estradiol administered *in vivo* may render these experiments uninterpretable. In contrast, if the pathophysiology mediating

AI-induced hypernociception were known, the contribution of estrogen signaling could be addressed by tissue-specific modulation of these pathways. Therefore, our future experiments will focus on determining whether AIs enhance the excitability of sensory neurons or alter their sensitivity to inflammatory mediators such as ATP.

To address whether AIs induce nociceptor sensitization, our initial experiments have focused on determining the direct effect of letrozole treatment on sensory neurons using a primary cell culture model. In these experiments, we have demonstrated that letrozole *per se* does not alter release of the neuropeptide iCGRP under basal conditions or during capsaicin or high potassium-stimulated release of iCGRP.

To support our release data, we conducted studies to identify whether aromatase was localized to neurons or non-neuronal cells. Our results suggest aromatase expression is localized to neurons, which is consistent with a previous study characterizing aromatase expression in whole DRGs [Schaeffer Glia 2009]. To further evaluate aromatase expression in sensory neurons in vitro and in vivo, we are conducting immunocytochemistry and immunohistochemistry studies to determine whether aromatase is co-expressed with several proteins that functionally segregate sensory neurons. These include calcitonin gene-related peptide, TRPV1, and purinergic receptors. These studies will provide important data to evaluate results of our functional studies.

Finally, in preparation for experiments to address whether AI-treatment alters the release of neuropeptides from nociceptor central terminals, I have re-established a working model to measure iCGRP release from rat spinal cord slices. We have validated the model using historic controls, including iCGRP release induced by capsaicin and augmentation of capsaicin-evoked iCGRP release following exposure of the spinal cord tissue to carbaprostacyclin.

In conclusion, the experiments presented here build on our prior data describing a preclinical model of AI-induced pain and provide additional observations as to a potential underlying mechanism. Further work is needed to establish that AI-induced hypernociception selectively alters purinergic signaling and that these results translate to the human condition. However, mechanistic results presented here may be used to rationally select or develop novel therapeutics for relief of AI-induced pain, such as purinergic receptor antagonists currently in development for pain.

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APPENDICES

None

SUPPORTING DATA

None