Published in final edited form as:

Eur J Pain. 2013 February ; 17(2): 174–184. doi:10.1002/j.1532-2149.2012.00183.x.

Modulation of temporomandibular joint nociception and inflammation in male rats after administering a physiological concentration of 17 β -estradiol

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Abstract

Background—Previous studies have shown 17 β -estradiol will reduce temporomandibular joint (TMJ) inflammation and hypersensitivity in female rats. Although, male rats contain significant amounts of estradiol it was unknown whether a physiological concentration of 17 β -estradiol would attenuate male TMJ inflammation and nociception.

Methods—Intact and castrated rats were given a physiological concentration of estradiol to examine first, if estradiol will affect male TMJ nociception/inflammation and second, if administration of estradiol would act synergistically with endogenous male hormones to attenuate TMJ nociception. The hormonally treated rats were given TMJ injections of complete Freund's adjuvant (CFA) and then nociception was measured using a validated method in which a lengthening in meal duration is directly correlated to the intensity of deep TMJ nociception. Inflammation was assayed by quantitating pro-inflammatory gene expression.

Results—Meal duration was significantly lengthened after TMJ CFA injection and this lengthening was significantly attenuated in the castrated but not intact males after administering a physiological concentration estradiol. A physiological concentration of 17 β -estradiol also significantly increased IL-6 expression in the inflamed TMJ of castrated males while 17 β -estradiol did not alter IL-1 β , CXCL2 and CCL20 expression. Castration increased pro-inflammatory mediators IL-6, IL-1 β and CXCL2 suggesting male sex hormones were anti-inflammatory. CGRP in the trigeminal ganglia was unchanged.

Conclusions—Similar to females, male rats with TMJ inflammation showed a reduced nociceptive response after treatment with a physiological concentration of estradiol suggesting the effects of estradiol treatment were not constrained by organizational processes in the males.

Introduction

Women seek treatment for temporomandibular joint (TMJ) pain more often than men, to the extent that they comprise over three-fourths of the clinical cases (LeResche 97). Moreover, women appear to exhibit more severe symptoms of TMJ disorders (Fillingim and Ness 00; Shinal and Fillingim 07). The peak incidence of TMJ disorders occurs during the childbearing years (Goulet et al 95) and varies in intensity over the menstrual cycle

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Conflicts of interest: None declared.

Authors Contributions

Dr. Kramer and Dr. Bellinger both participated in planning the experiments, as well as, analyzing and interpreting the data. Both authors also contributed in writing the manuscript.

(Suenaga et al 01; LeResche et al 03b). During pregnancy, when the concentration of gonadal hormones are increasing reported TMJ pain decreases (LeResche et al 05). In humans, low estrogen levels observed during the late luteal phase and during menstruation correlate to heightened pain responses in individuals with TMJ disorders (LeResche et al 03a). In a human study 17 β -estradiol decreased sensitivity to noxious subcutaneous stimuli over the TMJ region (Smith et al 06) consistent with the idea that either low estrogen or rapid changes in estrogen concentration result in an increase in TMJ pain in women.

Behavioral studies have shown that administering 17 β -estradiol will reduce TMJ nociception in female rats (Clemente et al 04; Arthuri et al 05; Favaro-Moreira et al 09). Consistent with these results, neuronal responses after TMJ stimulation can be reduced by estrogen treatment (Tashiro et al 11). To date, only non-orofacial nociceptive responses have been measured after the estradiol concentration in male rats was increased to a level equivalent to that found in female rats (Aloisi and Ceccarelli 00; Lawson et al 10; Hubscher et al 10). In a orofacial study, a supra-physiological concentration of estradiol showed there was no affect on TMJ nociception but this level of estrogen did reduce inflammation in a male rat (Fischer et al 07; Torres-Chavez et al 12). Recently our lab reported that inflammatory TMJ nociception was attenuated in ovariectomized female rats when the plasma concentration of 17 β -estradiol was 80 pg/ml (i.e., proestrus concentration) versus 20 pg/ml (i.e., diestrus concentration) but our previous work did not address whether the attenuating effect of estradiol was sex-specific (Kramer and Bellinger 09). Males contain significant levels of estradiol, levels similar to female rats in diestrus (Craft et al 04) but it was unknown what a physiological concentration of estradiol would have on a male with TMJ pain.

To address this question we asked first, does a physiological concentration of 17 β -estradiol alter the orofacial nociceptive and inflammatory response of males and second, do endogenous male sex hormones act synergistically with estradiol to reduce the orofacial nociceptive response. To address these questions intact and castrated male rats were given a physiological concentration of 17 β -estradiol equivalent to that found in female rats during proestrus and TMJ nociception and inflammatory mediators were measured.

Materials and Methods

Male (250 grams) Sprague-Dawley rats from Harlan (Houston, TX) were kept on a 14:10 light/dark cycle with lights on at 06:00 hours. The rats were 250 grams at the start of the experiments; this rat strain has the capacity to breed at this weight. The animals were housed individually in computerized feeding modules (Med Assoc. Inc., East Fairfield, VT) and given food and water ad libitum. They were acclimated to the surroundings for a week before surgery. All animal experiments were approved by the Baylor College of Dentistry Institutional Animal Care and Use Committee in accordance with the guidelines of the USDA and National Research Council's "Guide for Care and Use of Laboratory Animals" and the International Association for the Study of Pain (IASP).

Castration and artificial estrous cycle using 17 β -estradiol

Before surgery the rats were anesthetized with ketamine (52 mg/kg) and xylazine (5.4 mg/kg). Using sterile technique, a portion of the rats were castrated or sham operated and then the castrated and intact male rats were implanted with 28-day Alzet mini-osmotic pumps that were primed according to the manufactures directions (Durect Corporation, Cupertino, CA). Upon implantation the pumps were immediately dispensing 6 μ l/day of the vehicle polyethylene glycol (no 17 β -estradiol replacement control group) or 750 ng/6 μ l/day of 17 β -estradiol benzoate (Sigma, St. Louis) (Kerins et al 03; Guan et al 05). This dose of 17 β -estradiol benzoate produces plasma 17 β -estradiol concentration of approximately 10 pg/ml

(Guan et al 05); a concentration similar to that reported in diestrus-1, diestrus-2, estrus and metestrus (Kalra and Kalra 74; Butcher et al 74; Butcher et al 78). Five days after pump implantation, the rats receiving 17β -estradiol replacement were injected subcutaneously at 12:00 with 2.5 μ g of 17β -estradiol benzoate in 0.1 ml of sesame oil; this amount generated 80 pg of 17β -estradiol per ml of plasma in female rats (Kramer and Bellinger 09). Injection of 2.5 μ g 17β -estradiol increased attenuation of the pain response in a previous study (Kramer and Bellinger 09). At proestrus a rat's plasma estradiol is 80 pg/ml (Butcher et al 74; Butcher et al 78). This subcutaneous injection of 17β -estradiol benzoate was repeated every five days for three cycles. The control rats (polyethylene glycol pumps) received subcutaneous oil injections.

Assay for plasma estradiol

In preliminary studies the plasma concentration of 17β -estradiol was measured every 3 hours after injecting intact and castrated male rats with 2.5 μ g of 17β -estradiol. All rats were previously implanted with osmotic pumps dispensing 750 ng/6 μ l/day of 17β -estradiol. For these measurements rats were removed from their cage and quickly sacrificed by decapitation and trunk blood was collected for radioimmunoassay. 17β -estradiol was quantitated in the blood plasma by first, vigorously mixing 0.5 ml of plasma with 5 ml of ether. Second, the solution was frozen in an ethanol, dry ice bath and the ether phase decanted. The ether phase contains the lipid soluble hormones. The ether was evaporated and the remaining residue suspended in radioimmunoassay buffer supplied by the manufacturer and assayed according to the manufactures directions (Diagnostic Systems Laboratories, Webster, TX).

Nociceptive Assay

In the present study, meal duration was used as a method of measuring the magnitude of deep TMJ nociception (Kerins et al 03; Kerins et al 05; Kramer and Bellinger 09) in castrated and intact male rats given vehicle or 17β -estradiol. To measure meal duration rats were housed individually in 32 sound-attenuated chambers equipped with photobeam computer-activated pellet feeders. The rats were given 45 mg rodent chow pellets (Product No. FO 165, Bioserv, Frenchtown, NJ). When a rat removes a pellet from the feeder trough a photobeam placed at the bottom of the trough is no longer blocked, signaling the computer to drop another pellet, record the date and time, and keeps a running tally of the total daily food consumption. The computer record of pellets dropped over time allows for the meal patterns such as food intake, meal size, meal number and meal duration to be calculated using proprietary software. The meal size, meal number and food intake did not change significantly after injecting 15 μ g of CFA (data not shown). In contrast meal duration did change significantly after a 15 μ g CFA injection. The fact that meal duration changed and the other meal parameters did not change was consistent with previous results demonstrating that meal duration is a continuous non-invasive biological marker of TMJ nociception (surface and deep) in undisturbed male and female rats (Kerins et al 03; Kerins et al 05; Kramer and Bellinger 09).

Complete Freund's adjuvant (CFA) treatment

Prior to the TMJ saline or CFA injections, the rats were removed from their cages and anesthetized with a 5% flow of isoflurane gas. Saline 0.9%, (30 μ l) or CFA (30 μ l paraffin oil/15 μ g *Mycobacterium tuberculosis*) was injected bilaterally into the superior joint space of the TMJ on the day 2.5 μ g of 17β -estradiol was subcutaneously injected (i.e., boost). The TMJ injections were given at 13:00 h, which was one hour after the 17β -estradiol boost. Following the TMJ injections and removal from anesthesia, the rats began freely moving within five minutes. They were returned to their feeding modules, and meal duration was

recorded in the undisturbed animals over the following 24 hours. All the pre-injection data was collected for each group five days earlier.

Molecular assays

The day following TMJ saline or CFA injection the rats were removed from their cage and quickly sacrificed by decapitation, trunk blood was collected for radioimmunoassay and the heads were submerged in an ice bath until dissection. To minimize the release of corticosterone and the effects of corticosterone on the levels of cytokines decapitation was necessary (Schultz et al 79; Vahl et al 05). TMJ and trigeminal ganglia tissues were dissected to a standard size, rapidly frozen in liquid nitrogen and stored at -80°C for later molecular assays. TMJ dissection included removing retrodiscal, disc, and synovial tissue. The TMJ tissue from one side of the head was homogenized in buffer (75 mM potassium acetate pH 7.4, 300 mM NaCl, 10 mM EDTA, 0.25% Triton X-100, protease inhibitors). The trigeminal ganglia were homogenized in an acid buffer per manufacturer's directions and analyzed for calcitonin gene-related peptide (CGRP) using a radioimmunoassay (Phoenix Pharmaceutical, Mountainview, CA). CGRP was studied because inflammation causes an increase in trigeminal ganglia CGRP (Hutchins et al 00; Spears et al 05) and an increase in trigeminal ganglia CGRP has been linked to craniofacial nociception (Ambalavanar et al 06) suggesting CGRP can be used as a marker for craniofacial nociception. Interestingly, estradiol has been shown to stimulate CGRP expression in the dorsal root ganglia of female rats (Gangula et al 00) consistent with the idea that estrogen can alter the nociceptive response after TMJ CFA injection by modulating expression of CGRP in the trigeminal ganglia.

Using the TMJ homogenate we chose to quantitate IL-1 β , IL-6, CXCL2 and CCL20 using ELISA (R&D Systems, Minneapolis, MN) because one, elevated amounts of cytokines IL-1 β , IL-6 and chemokines CXCL2 and CCL20 negatively affect TMJ disease (Kubota et al 98; Kopp 98; Takahashi et al 98; Ruth et al 03; Tominaga et al 04; Ogura et al 07) two, previous studies have shown that CFA will increase the concentration of these molecules in the TMJ (Kerins et al 03; Longoria et al 06; Kramer and Bellinger 09) and three, sex hormones can effect expression of IL-1 β , IL-6, CXCL2 and CCL20. In animal models inflammatory arthritis, including TMJ arthritis, has been associated with higher levels of IL-1 β , IL-6, CXCL2 and CCL20 (Kagari et al 02; Kerins et al 03; Ruth et al 03; Kerins et al 05; Cuzzocrea et al 06; Kramer and Bellinger 09; Tissi et al 09). IL-1 β and IL-6 play a role in TMJ arthritis (Alstergren et al 98; Kopp et al 05; Lai et al 06) and CXCL2 is known to function in cellular migration in inflamed tissue (Verri, Jr. et al 07). CCL20 is elevated in human arthritic tissues and its expression in synovial fibroblasts has been associated with the production prostaglandins, pro-inflammatory cytokines and T cell recruitment (Chabaud et al 01; Ruth et al 03; Kawashiri et al 09; Alaaeddine et al 11), moreover blockade of CCL20 resulted in reduced arthritic symptoms (Hirota et al 07). Estradiol treatment will enhance expression of IL-6 in the TMJ (Torres-Chavez et al 11) and alter IL-1 β and CXCL2 production in monocytes (Polan et al 88; Hu et al 88; Polan et al 89; Huang et al 08). Estradiol will also alter expression of CCL20 in the trigeminal ganglia of rats (Puri et al 06). Because these molecules are present in a diseased TMJ and can be modulated by estrogen we expected them to have a role in the inflammatory and nociception response.

Each homogenized sample was also tested for total protein content by a standard BCA assay (Thermo Scientific, Rockford, IL); the cytokines, chemokines and CGRP data were expressed as pg/mg total protein. Samples from each animal were completed in duplicate for each type of assay.

Statistics

A change in the concentration of 17 β -estradiol in the plasma (dependent variable) was analyzed by ANOVA using the independent variables of whether the rat was intact or castrated and if the rat received vehicle (i.e., polyethylene glycol) or 17 β -estradiol and time. The hormone effect on meal duration (dependent variable) was determined by ANOVA using the independent variables; intact or castration, vehicle or 17 β -estradiol treated, CFA or saline TMJ injected and time (i.e., previous cycle and post TMJ injection). Also, the CFA effect on meal duration was determined on the day after TMJ injection using ANOVA (independent variables; intact or castration, vehicle or 17 β -estradiol treated, CFA or saline TMJ injected (ABstat software, V1.94). The dependent variables for the molecular studies were CGRP, IL-1 β , IL-6, CCL20 or CXCL2 concentration. The concentration of these molecules was analyzed using ANOVA (independent variables; intact or castration, vehicle or 17 β -estradiol treated, CFA or saline TMJ injected). If a significant main effect was found the data was further analyzed using Tukey's post-hoc test.

Results

Implanting osmotic pumps containing 17 β -estradiol produced a baseline 5–10 pg/ml of estradiol in the intact male Sprague-Dawley rats and produced 10–15 pg/ml of estradiol in the castrated male rats (Fig. 1); this difference in plasma estradiol concentration was not significantly different between the two groups ($p=0.4$). To simulate the concentration of estradiol present during the proestrus phase an additional amount of 17 β -estradiol was injected every 5th day. Injection significantly increased the plasma concentration of 17 β -estradiol over time ($p<0.01$) but there was no significant difference in 17 β -estradiol between the intact and castrated rats. Plasma 17 β -estradiol for intact cycling female rats was overlaid on Figure 1.

No significant difference in the meal duration was found among any of the groups at pre-injection (Fig. 2). CFA injection did significantly lengthened meal duration versus the saline injected animals in each treatment group (Fig. 2). Castration did not significantly alter the meal duration [$F(1,85)=1.0$, $P=0.3$], but the subcutaneous administration of 17 β -estradiol did have a significant anti-nociceptive effect, i.e., shortening the meal duration in the castrated males when compared to CFA treated castrated rats receiving vehicle (Fig. 2). A significant interaction was observed between castration and estradiol treatment [$F(1,85)=6.2$, $P=0.01$].

Injection of CFA into the TMJ significantly increased cytokines IL-6 and IL-1 β in the TMJ tissue (Fig. 3). Administration of 17 β -estradiol increased IL-6 levels further but only in the castrated males (Fig. 3a). While 17 β -estradiol had no effect on the amount of IL-1 β in the TMJ, [$F(1,68)=0.002$, $P=0.95$], castration increased IL-1 β in the inflamed TMJ when compared to intact controls receiving 17 β -estradiol or its vehicle (Fig. 3b). There was a significant interaction between castration and inflammation for IL-6 and IL-1 β , [$F(1,71)=3.7$, $P=0.05$] and [$F(1,68)=4.95$, $P=0.03$] respectively. No other interactions were significant.

17 β -estradiol did not affect the amount of CXCL2 in the inflamed TMJ (Fig. 4a), [$F(1,70)=0.003$, $P=0.95$] and there was no significant interaction between castration and estradiol treatment or estradiol treatment and inflammation. CFA injection resulted in an increased amount of CXCL2 ($p<0.01$) in the TMJ of both castrated and intact males (Fig. 4a) with an interaction between castration and inflammation [$F(1,70)=6.5$, $P=0.02$]. CFA injection significantly increased CCL20 levels in the TMJ (Fig. 4b). Neither castration nor 17 β -estradiol significantly altered the amount of CCL20 in the TMJ tissues (Fig. 4b) but

there was a significant interaction between estradiol administration and inflammation [$F(1,69)=4.65$, $P=0.03$].

Measurements of CGRP in the trigeminal ganglia indicated that neither 17β -estradiol nor castration affected the total amount of this molecule (Fig. 5).

Discussion

The present data demonstrates that giving castrated male rats a physiological concentration of 17β -estradiol can attenuate an orofacial nociceptive response. Like the males, estrogen has been shown to attenuate orofacial nociception in female rats after injecting the TMJ with CFA (Kramer and Bellinger 09), formalin or glutamate (Clemente et al 04; Fischer et al 08). Interestingly, administration of a supra-physiological concentration of estrogen did not affect TMJ nociception of castrated male rats (Fischer et al 07) suggesting it was important that estrogen be given at a physiological dose. In support of the idea that a physiological concentration of estrogen can reduce nociception, studies in female rat models showed non-orofacial nociceptive responses can be reduced after administering 17β -estradiol (Craft et al 04). Estradiol has also been shown to modulate non-orofacial nociceptive behavior in male rats but the effects depended on the behavioral response being measured. For example, a physiological concentration of estradiol will increase the nociceptive response for one type of behavior but will be anti-nociceptive for a different behavior (Aloisi and Ceccarelli 00; Lawson et al 10; Hubscher et al 10) indicating that not only should the dosage of hormone be closely monitored but one must note the type of pain response being measured.

The higher prevalence of TMJ disorders in females versus males may be due to the hormonal fluctuations observed in women. In women the pain threshold has been reported to change with the stage of the menstrual cycle (Ring et al 09). In a human study pain ratings were higher in women having a low concentration of estrogen versus women with a high amount of estrogen (Smith et al 06). Consistent with this idea, a (lower) diestrus concentration of estradiol was less protective of the nociceptive response than a (higher) proestrus concentration in female rats (Kramer and Bellinger 09). Once these hormonal fluctuations are reduced, such as post-menopausally, the prevalence of TMJ disorders are also reduced (Von Korff et al 88; Carlsson and Le Resche 95) consistent with the idea fluctuating hormones increase TMJ pain in women. Oral contraceptives studies show conflicting results with respect to TMJ pain (Marbach et al 88; LeResche et al 97), these conflicts could result from the pattern and concentration of hormones administered. The higher prevalence of TMJ disorders in females may also be due to male hormones being protective against pain (Fischer et al 07). Males show higher opioid activation and a reduced pain response versus females (Zubieta et al 02). Testosterone can reduce the nociceptive response caused by adjuvant-induced arthritis (Harbuz et al 95), although the type of pain induced and the location of painful condition can affect the ameliorating action (Ali et al 95; Cicero et al 96).

In contrast to castrated males the intact male rats were not affected by 17β -estradiol treatment suggesting this concentration of estradiol does not act synergistically with male hormones to reduce the nociceptive response. If estradiol and the endogenous male hormones were synergistic the nociceptive response should have been attenuated to a greater extent in the intact males versus the castrated males. Moreover, the observation that a surge in 17β -estradiol attenuates nociception in only castrated but not intact males suggests male hormone(s) antagonized the anti-nociceptive actions of 17β -estradiol. An alternative explanation however, is that the higher amount of estradiol in the intact males (~140 pg/ml), after injection, contributed to the lack of attenuation. It would be interesting to inject intact males with only enough estradiol to produce a surge of 70–80 pg/ml, equivalent to the

castrated males, and observe the nociceptive response. If our results were due to the high amount of estradiol (i.e., 140 pg/ml) and not antagonism of the 17β -estradiol pathway then a lower amount of 17β -estradiol (i.e., 70–80 pg/ml) should result in attenuation. If the 70–80 pg/ml concentration of estradiol does not attenuate the nociceptive response in the intact males then antagonism by a male hormone would be a more consistent explanation of the results.

Castration did not affect the CFA induced nociceptive response, consistent with earlier findings that castration does not affect the nociceptive response resulting from injecting 1.5% formalin into the TMJ (Fischer et al 07) and findings that castration of male rats does not alter their response to inflammatory injury of the face (Pajot et al 03). These data are also consistent with the lack of an anti-nociceptive effect observed in other body joints (Ali et al 95; Cicero et al 96) but contrary to a study where testosterone reduced nociceptive responses caused by adjuvant-induced arthritis (Harbuz et al 95). This inconsistency may be explained by the dosage of inflammatory agent administered. For example, while testosterone was not protective against 1.5% formalin in the TMJ, it was protective against a 0.5% dose (Fischer et al 07).

Previously we showed that a proestrus concentration of 17β -estradiol was associated with an upward trend in TMJ IL-6 (Kramer and Bellinger 09). Here we observed the highest IL-6 expression in castrated rats given estradiol but the highest amount of 17β -estradiol was actually observed in the intact male rats and not the castrated rats. Two potential explanations for this result are either IL-6 expression is maximal at a specific 17β -estradiol concentration (i.e., between 20–140 pg/ml) as has been shown for IL-1 (Polan et al 90). Or an endogenous male hormone attenuated IL-6 expression in the intact males, similar to the findings of Torres-Chavez et al., (Torres-Chavez et al 11) who also found that IL-6 was elevated in female rats when compared to intact male rats. Interestingly, IL-6 levels were highest and the nociceptive response was lowest in the TMJ of castrated males given a proestrus concentration of 17β -estradiol. Such a result is consistent with the idea that IL-6 had a role in the protective effects of estradiol.

Alternatively, estrogen could alter TMJ nociceptive responses by mechanisms that are not related to the cytokine levels. For example, a reduced nociceptive response in female rats has been shown to involve kappa opioid receptors in the trigeminal ganglia and a peripheral nitric oxide-cyclic guanosine monophosphate signaling pathway (Clemente-Napimoga et al 09; Favaro-Moreira et al 09). Within the upper spinal cord and medulla region estrogen has been shown to alter neuronal activity (Tashiro et al 07; Tashiro et al 11) suggesting estrogen can induce changes in the trigeminal ganglia, spinal cord and medulla to effect the way neurons process pain signals after estradiol administration.

Inflammation induced an increase in TMJ IL-1 β and CXCL2 in the intact and castrated rat receiving vehicle or 17β -estradiol. This is in contrast to studies demonstrating reduced cytokine levels in castrated rats given estrogen (Torres-Chavez et al 12). IL-1 β and CXCL2 were significantly higher in the castrated groups whether given 17β -estradiol or vehicle. Thus, the lack of testosterone enhances IL-1 β and CXCL2 in the inflamed TMJ at 24 hours. The current 24 hour IL-1 β data contrasts with the 45 minute sampling data following TMJ injection of 1.5% formalin (Torres-Chavez et al 11), where males had significantly higher levels of IL-1 β than female rats. The method of inducing inflammation or the time of sampling may have been responsible for the differing results. The present data do suggest that changes in TMJ IL-1 β or CXCL2 are not responsible for the attenuation of TMJ nociception following a proestrus-like rise in 17β -estradiol. The results also suggest the immune response in the TMJ can be modulated by testosterone.

In the trigeminal ganglia of a rat CCL20 was reduced by estrogen treatment (Puri et al 06), but it was unknown what effect estrogen has on CCL20 in the TMJ. Here we demonstrate that CCL20 in the TMJ is not significantly affected by 17 β -estradiol treatment.

The CGRP data is the same as what was observed in females, showing no change in the level of total protein or mRNA for the various treatment groups (Puri et al 05; Kramer and Bellinger 09). This result does not exclude a potential function of CGRP in the trigeminal ganglia, since we did not measure release properties during treatment or changes in expression from neurons that project to the TMJ. Alternatively, the lack of change in CGRP expression could suggest that estrogen modulates the nociceptive response by effecting cellular function in the upper spinal cord/medullary region or even the thalamus and cortex (Bereiter et al 05; Tashiro et al 07; Okamoto et al 08).

In conclusion, a rise in plasma 17 β -estradiol to 80 pg/ml attenuated TMJ nociception in castrated male rats and these findings are very similar to previous data in female rats. IL-6 was elevated in castrated rats after estrogen treatment, which could be protective and possibly reduce nociception by increasing extravasation. While castration caused an increase in IL-1 β and CXCL2, this increase did not seem to relate to the anti-nociceptive effects of 17 β -estradiol. The data suggest that estradiol's attenuation of the nociceptive response was not sex-specific at least in the absence of endogenous male hormones.

Acknowledgments

Funding Sources: This study was funded through NIH/NIDCR grants DE016059 to LLB and DE15372 to PRK

The authors thank Connie Tillberg and Vanessa Winger for her technical expertise.

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Bulleted Statements**What's already known about this topic?**

- The sex specific effects of estradiol on non-facial pain has also been studied in males.

What does this study add?

- No study has looked at the sex specific effect of estradiol on orofacial pain using a physiological concentration of hormone
- Estradiol's effect on orofacial pain and inflammation in males was analyzed and any potential synergism between an endogenous male hormone(s) and the administered 17 β -estradiol was investigated.

17 β -ESTRADIOL

Figure 1. The concentration of 17 β -estradiol in castrated and intact male Sprague Dawley rats supplemented with estradiol

In male rats an artificial estrous cycle was produced by implanting osmotic pumps releasing a constant amount of 17 β -estradiol to produce a baseline level of estradiol and a boost of estrogen was given by subcutaneously injecting 17 β -estradiol every 5th day. At 3 hour intervals the amount of hormone was measured after 17 β -estradiol injection in both castrated male (●) and intact male (○) rats. The plasma concentration of 17 β -estradiol for intact female rats (□) is redrawn with permission from Butcher et al., 1974. M = Midnight. Data were from 6 male rats per treatment group and plotted as mean $\pm$ SEM.

Figure 2. A graph showing the 24-hour meal duration for intact (INT) or castrated (CAST) male rats

The INT and CAST male rats were given Alzet pumps dispensing either the vehicle polyethylene glycol (PEG) or 17 β -estradiol (E2). In the rats with a pump dispensing E2 a subcutaneous injection of 17 β -estradiol benzoate in sesame seed oil was administered every 5 days to simulate the proestrus surge. For the rats implanted with a pump containing PEG a subcutaneous injection of sesame seed oil was completed every 5 days. Data from the second subcutaneous injection, 5 days prior to when they were given temporomandibular joint (TMJ) injection, is termed the PREVIOUS CYCLE. Data 24 hours following the third subcutaneous injection and TMJ injection of saline or CFA is termed POST SAL/CFA. When comparing the SAL and CFA groups, CFA significantly lengthened meal duration, "a" = $P < 0.01$. The CAST+PEG+CFA group had a significantly longer meal duration than the CAST+E2+CFA group (* = $P < 0.05$). The duration of the meals for 10 rats was collected and plotted as a mean $\pm$ SEM.

Figure 3. Cytokines in the TMJ of cycling, castrated rats and in intact rats

Treatment groups are outlined in Figure 2. In panel a the amount of IL-6 and in panel b the amount of IL-1 β was measured in the TMJ tissue (i.e., retrodiscal, disc and synovium) by ELISA 24 hours after injecting saline or complete Freund's adjuvant (CFA) into the TMJ. Values for IL-6 and IL-1 β were calculated per mg of total protein in the tissue. Data were

from 10 animals per treatment group and plotted as mean $\pm$ SEM. When comparing the SAL and CFA groups, CFA significantly increased the amount of cytokine in the TMJ. “a” = $P < 0.01$. * = $p < 0.05$.

Figure 4. Chemokines in the TMJ of cycling, castrated rats and in intact rats

Treatment groups are outlined in Figure 2. In panel a the concentration of CXCL2 and in panel b the amount of CCL20 in the TMJ tissue was assayed by ELISA 24 hours after injecting saline or complete Freund's adjuvant (CFA) into the TMJ. Values for CXCL2 and CCL20 were calculated per mg of total protein in the tissue. Data were from 10 animals per treatment group and plotted as mean $\pm$ SEM. When comparing the SAL and CFA groups,

CFA significantly increased the amount of chemokines in the TMJ. “a” = $P < 0.01$. ** = $p < 0.01$.

CGRP

Figure 5. The amount of calcitonin gene-related peptide (CGRP) in the trigeminal ganglia was measured 24 hours after saline or CFA TMJ injections by radioimmunoassay. Treatment groups are outlined in Figure 2. Values for the CGRP concentration were calculated per mg of total protein in the tissue. Data were from 10 animals per treatment group and plotted as mean $\pm$ SEM.