Temporomandibular disorders (TMDs) are a collection of common conditions that affect the temporomandibular joint (TMJ) and/or the muscles of mastication and are often associated with chronic and debilitating pain. The prevalence, severity, and duration of TMD pain are greater in women than in men, and TMD pain primarily affects women during their reproductive years (8). Furthermore, women who take exogenous estrogens have an increased risk of referral for treatment of TMD pain (23). These observations suggest a potential role of TMD pain in the presence of overt inflammation. Importantly, our results may help explain both the higher prevalence and severity of temporomandibular disorder pain in females than males.

Testosterone and estrogen have opposing actions on inflammation-induced plasma extravasation in the rat temporomandibular joint

Flake, Natasha M., Tracey O. Hermanstyne, and Michael S. Gold. Testosterone and estrogen have opposing actions on inflammation-induced plasma extravasation in the rat temporomandibular joint. Am J Physiol Regul Integr Comp Physiol 291: R343–R348, 2006. First published February 9, 2006; doi:10.1152/ajpregu.00835.2005.— The present study was designed to test the hypothesis that estrogen exacerbates inflammation of the temporomandibular joint (TMJ). Evans blue dye was used to quantify plasma extravasation (PE) around the rat TMJ. In an initial set of experiments, TMJ PE was compared in naïve intact male and female rats, as well as in both groups after complete Freund’s adjuvant (CFA)-induced inflammation of the TMJ. In contrast to our hypothesis, TMJ PE was significantly greater in both naïve and CFA-inflamed male rats than in females. To determine whether these differences were due to gonadal hormones, four additional groups of rats were studied: gonadectomized (Gx) males and females, Gx males with chronic testosterone (T) replacement, and Gx females with chronic estrogen (E) replacement. The sex difference in baseline TMJ PE appeared to reflect the actions of T. However, in the presence of TMJ inflammation, T augmented TMJ PE in males, while E attenuated TMJ PE in females. Changes in PE were also assessed in the contralateral TMJ. Results from this analysis indicated that there is a transient contralateral increase in PE in females but not males. Given that there is an inverse relationship between PE and joint damage, our results suggest that testosterone may mitigate, but estrogen may exacerbate, TMJ damage, particularly in the presence of overt inflammation. Importantly, our results may help explain both the higher prevalence and severity of temporomandibular disorder pain in females than males.

One potential mechanism by which estrogen may contribute to TMD pain is by modulating the inflammatory process that is associated with damage to the TMJ. The present study was therefore designed to test the hypothesis that estrogen exacerbates inflammation of the TMJ. To test this hypothesis, we assayed plasma extravasation (PE) in the TMJ of intact and gonadectomized male and female rats receiving chronic gonadal hormone replacement in the presence and absence of TMJ inflammation.

MATERIALS AND METHODS

All experiments were approved by the University of Maryland Dental School Institutional Animal Care and Use Committee and performed in accordance with standards promulgated by the International Association for the Study of Pain and the National Institutes of Health on the care and use of laboratory animals. Male and female Sprague-Dawley rats (Harlan, 170–250 g at the time of arrival to the animal facility) were used for all experiments.

Gonadectomy and hormone replacement. Rats were anesthetized with rat cocktail (55 mg/kg ketamine, 5.5 mg/kg xylazine, and 1.1 mg/kg acepromazine). Ovaries were accessed via a lateral approach, tied off, and excised. A pellet of 17β-estradiol (E; 0.1 mg/pellet, 21-day release, Innovative Research of America, Sarasota, FL) or vehicle was inserted subcutaneously between the shoulder blades (12). Testes were accessed through the lower abdomen, tied off, and excised. A Silastic tube (0.075 in ID, 0.125 in OD, 1.5 in length) filled with testosterone or left empty (blank), preoxygenated overnight in saline, was inserted subcutaneously between the shoulder blades (32). Intramuscular injections of buprenorphine (0.03 mg/kg) and penicillin G (10,000 units/kg) were administered postoperatively.

Induction of inflammation. Rats were anesthetized with rat cocktail. Unilateral inflammation was induced by injecting complete Freund’s adjuvant (CFA; 50 μl; 1:1 in saline; Sigma, St. Louis, MO) into the TMJ as previously described (12). CFA or saline was injected slowly over a period of 2 min. The CFA/saline injection into the TMJ occurred 16–17 days after the gonadectomy and hormone replacement.

Determination of plasma extravasation. Rats were anesthetized with rat cocktail. Evans blue dye (Sigma; 50 mg/kg of a 50 mg/ml solution) was injected into the femoral vein. Ten minutes later, rats were perfused transcardially with 300 ml of PBS to flush Evans blue from the vasculature. Rats were then decapitated, and TMJs were collected. TMJs were dissected to a standardized size, such that the dissected joint measured 8 mm2 when positioned with the zygomatic process facing upward. Healthy muscle was removed, leaving primar-
RESULTS

Sex differences in resting and inflammation-induced increases in TMJ PE. We first sought to determine whether there was an influence of sex on resting and inflammation-induced increases in TMJ PE. Resting TMJ PE levels were assessed in 6 naïve intact male and 6 naïve intact female rats (Fig. 1A). Mean tissue content of Evans blue (μg/mg dry weight of tissue) was significantly larger in males than in females. Within animal comparison of TMJ PE levels (comparing right and left TMJ with paired Student’s t-test) revealed no significant differences between joints in naïve animals (data not shown). The time course of the CFA-induced increase in TMJ PE is shown in Fig. 1B; data at each time point were pooled from five or six animals. There were significant main effects due to sex and time. TMJ PE was increased in both groups more than fourfold above resting levels within 12 h. By 24 h post-CFA injection, TMJ PE had decreased in both groups. However, the decrease was greater in female than male rats. Furthermore, while TMJ PE was significantly elevated above resting levels at 72 and 144 h post-CFA in both groups, TMJ PE levels were consistently greater in male than in female rats.

PE in contralateral TMJ. There is evidence that inflammation of the rat TMJ results in bilateral activation of the trigeminal dorsal horn (36). There is also evidence from other somatic joints that there is a neurogenic component to inflammatory responses that arises in part from antidromic activity in nociceptive afferents driven by dorsal root reflexes (35). Importantly, bilateral increases in neuropeptides in TMJ periaxial tissues have been reported after a unilateral injection of adjuvant into the TMJ (7). Thus it is possible that a unilateral TMJ CFA injection results in a bilateral increase in PE. To assess this possibility, PE was assessed in TMJ contralateral to those obtained from intact male and female rats. Data are plotted in Fig. 2 with data from naïve animals replotted at time 0. There was a significant main effect due to sex, and a significant interaction between sex and time. This interaction was due to

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**Fig. 2.** TMJ contralateral to those inflamed with CFA also demonstrated a significant sex difference with Evans blue PE, greater, on average, in males than in females. Two-way ANOVA also revealed a significant interaction between sex and time, which reflected a significant elevation in PE in contralateral TMJ 12 h post-CFA injection in females. Contralateral TMJ were obtained from the same rats used to generate Fig. 1B. Data from naïve rats are plotted to facilitate comparisons.

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### Verification of hormonal treatment.

We were unable to determine plasma hormone levels of the rats in the present study, because the Evans blue dye interfered with the radioimmunoassay. Therefore, alternative means of verifying the efficacy of hormonal manipulations were used. In the female rats used in the present study, uterine weight was used as a measure of the presence or absence of estrogen. In a second group of 38 male and female rats treated identically to those used in the present study (intact male (n = 8) and female (n = 7), gonadectomized male (n = 7) and female (n = 4), and gonadectomized male (n = 5) and female (n = 8) receiving hormone replacement), plasma hormone levels were determined via radioimmunoassay at a commercial analytical lab (Ani Lytics, Gaithersburg, MD).

### Statistical analyses.

A Student’s t-test was used to assess the influence of sex on resting PE. The sex- and time-dependent effects of inflammation on resting PE. The sex- and time-dependent effects of inflammation on TMJ PE were assessed with a two-way ANOVA. A post hoc all-pairwise multiple comparisons were performed using the Holm-Sidak test. Pooled data are plotted as means ± SE. P < 0.05 was considered statistically significant.

### Materials and Methods

The amount of Evans blue dye extracted per joint was calculated based on a standard curve. Tissue was dried at 67°C. The magnitude of plasma extravasation (PE) was quantified as micrograms of Evans blue per milligram dry weight of tissue.

### Evans blue dye interference with radioimmunoassay.

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a significant increase in contralateral TMJ PE in females at 12 h post-CFA, but not in males.

**Testosterone augments TMJ PE in naïve rats.** To determine the influence of gonadal hormones on resting TMJ PE levels, four additional groups of rats were studied: 1) gonadectomized (Gx) males, 2) Gx males receiving testosterone (T) replacement, 3) Gx females, and 4) Gx females receiving estrogen (E) replacement. Four to six animals were used in each group. Mean Evans blue content in TMJ is plotted in Fig. 3. Data for intact animals were replotted for comparison. There was a significant main effect due to hormonal status in male but not female rats. TMJ PE in intact male rats or Gx male rats receiving T replacement was significantly greater than that in Gx male rats. Statistical comparisons between male groups indicated in Fig. 3 were the results of one-way ANOVA run-on data from naïve animals only.

**Testosterone augments and estrogen attenuates CFA-induced increases TMJ PE.** To determine the influence of gonadal hormones on the CFA-induced increase in TMJ PE, the same four additional groups of rats were studied 72 h after CFA injection. This time point was chosen because the greatest difference between males and females in CFA-induced PE was observed at 72 h. Five or six animals were used in each group. Mean Evans blue content in TMJ is plotted in Fig. 4. Data for intact animals were replotted for comparison. There was a significant (P < 0.01) main effect of hormonal status in both males and females. Post hoc analysis of male data revealed that TMJ PE was significantly greater in both intact and Gx + T groups than in the Gx group. Post hoc analysis of female data revealed that TMJ PE was significantly greater in both intact and Gx groups than in the Gx + E group.

PE data from TMJ contralateral to inflamed joints assessed at 72 h are plotted in Fig. 3. Assessment of the influence of gonadal hormones and inflammation on contralateral TMJ (i.e., two-way ANOVA of groups plotted with solid and hatched bars, Fig. 3) revealed a significant (P < 0.01) interaction between hormonal status and the presence of inflammation for both males and females. Post hoc analysis within males revealed that contralateral TMJ PE was significantly greater in Gx males, 72 h after inflammation of the ipsilateral TMJ, than in naïve Gx males. Post hoc analysis within females revealed that 72 h after CFA injection in the ipsilateral TMJ, contralateral TMJ PE was significantly lower in the Gx + E group than that in intact or Gx groups. For the sake of clarity, results of these post hoc comparisons are not indicated in the figure. The influence of chronic estrogen replacement in the presence of inflammation is different than in the absence of inflammation.

**Verification of estrogen treatment.** The uterine weight of intact (459 ± 31 mg, n = 6) and Gx + E rats (698 ± 27 mg, n = 5) was significantly greater than Gx rats (226 ± 16 mg, n = 5, P < 0.01). These uterine weights are similar to those from groups of rats in which both uterine weight and plasma estrogen concentrations were determined. Plasma hormone concentrations from rats treated identically to those used for PE experiments were as follows: Testosterone (T) levels were 2.66 ± 0.46 ng/ml (n = 8), 0 ng/ml (n = 7), and 4.55 ± 2.38 ng/ml (n = 5) in intact, Gx and Gx + T males, respectively. Estrogen (E) levels were 19.76 ± 3.32 pg/ml (n = 6), 0.51 ± 0.30 pg/ml (n = 4), and 62.22 ± 7.14 pg/ml (n = 8) for intact, Gx and Gx + E females, respectively.

**DISCUSSION**

The present study was designed to begin to test the hypothesis that estrogen contributes to the sex difference in the prevalence, severity, and duration of TMD pain by increasing inflammation associated with tissue damage within the TMJ. PE was used to assess the magnitude of the inflammatory response. In contrast to our hypothesis, we observed that both resting and CFA-evoked TMJ PE is greater in males than in females. These sex differences were eliminated with gonadectomy and reconstituted with hormone replacement.

As expected, an injection of CFA into the TMJ increased PE in all groups, an observation that is consistent with previous observations of the impact of inflammation in the TMJ. That is, CFA has been shown to increase Evans blue PE in the TMJ in male rats (36). Furthermore, CFA in the TMJ has been shown to produce histological changes indicative of inflammation (19, 20). Our results are also consistent with elevated levels of neuropeptides that promote PE (substance P, CGRP, and neu-
rokinin A) that have been observed in TMJ perfusates after injection of adjuvant into the TMJ (7, 8).

The greater resting PE observed in males appeared to reflect the actions of testosterone, as Gx reduced TMJ PE to levels observed in intact females, and this reduction was reversed by the exogenous administration of testosterone in Gx males. Testosterone influences processes that should both increase and decrease PE. For example, a testosterone-mediated inhibition of hypothalamic-pituitary-adrenal (HPA) axis output (17) or suppression of adrenal medullary output, resulting in lower plasma epinephrine levels (34), should contribute to an increase in resting PE. However, a testosterone-mediated suppression in the generation of proinflammatory cytokines from immune cells (26) should contribute to a decrease in resting PE. Thus results from the present study suggest that proinflammatory processes mediated by testosterone predominate in the rat TMJ.

The observation that estrogen had no detectable influence on resting TMJ PE was somewhat surprising in light of previous observations, indicating that like testosterone, estrogen may influence processes that may both increase and decrease PE. For example, estrogen may contribute to the suppression of PE by mediating an increase in resting HPA function (17), resulting in higher plasma corticosterone levels, or by suppressing the generation of proinflammatory cytokines from immune cells (33). Estrogen also appears to drive adrenal medullary activity, resulting in higher plasma concentrations of epinephrine (34), and epinephrine may both increase PE via α2-mediated effects or decrease PE via β2-mediated effects (10). The observation that neither gonadectomy nor estrogen replacement had any detectable influence on resting TMJ PE suggests that at least within the TMJ in the absence of inflammation, there is a balance between proinflammatory and anti-inflammatory influences of estrogen.

The sex difference in TMJ PE was maintained in the presence of persistent inflammation; on average, TMJ PE was lower in females than in males. By 72-h post-CFA injection, staining was observed in intact females, and this reduction was reversed by the exogenous administration of testosterone in Gx males. Testosterone influences processes that should both increase and decrease PE. For example, a testosterone-mediated inhibition of hypothalamic-pituitary-adrenal (HPA) axis output (17) or suppression of adrenal medullary output, resulting in lower plasma epinephrine levels (34), should contribute to an increase in resting PE. However, a testosterone-mediated suppression in the generation of proinflammatory cytokines from immune cells (26) should contribute to a decrease in resting PE. Thus results from the present study suggest that proinflammatory processes mediated by testosterone predominate in the rat TMJ.

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The sex difference in TMJ PE was maintained in the presence of persistent inflammation; on average, TMJ PE was lower in females than in males. By 72-h post-CFA injection, TMJ PE was >30% lower in females than in males. The sex difference at 72 h appeared to reflect both facilitative effects of testosterone and suppressive effects of estrogen. These results are similar to the effects of testosterone and estrogen in a model of acute neurogenic PE in the knee joint, where testosterone facilitates PE and estrogen suppresses it (14). Interestingly, at the earliest time point examined in the present study (i.e., 12 h post-CFA injection), TMJ PE was as large in females as in males. This observation was surprising in light of previous data, indicating that the influence of gonadal hormones on neurogenic PE in the knee joint was detectable within 10 min and persisted over the entire recording period (90 min) (14). Given that the mechanism underlying the actions of both testosterone and estrogen in the bradykinin model appears to be epinephrine release from the adrenal medulla, a sex difference was expected at 12 h post-CFA in the present study.

It is possible that our failure to detect a sex difference 12 h post-CFA is due to a “ceiling” effect, wherein the intensity of the inflammatory reaction at this time point was such that the ability to detect the “real” magnitude of TMJ PE was beyond the sensitivity range of our assay. An alternative explanation is the interaction between gonadal hormones and inflammation is dynamic, changing with time during persistent inflammation. Contralateral TMJ data support the latter possibility, given that there was a significant interaction between hormonal status and the presence of inflammation with respect to the magnitude of TMJ PE for both males and females. Additional support for the suggestion that there are time-dependent changes in interaction between gonadal hormones and the inflammatory response comes from the following observations: 1) persistent inflammation is clearly stressful, resulting in alterations in the HPA axis (18); 2) gonadal hormones influence the response to stress, with data from several studies indicating that the interaction between stress and gonadal hormones occurs at both the HPA and sympatho-adrenal axes (15, 30, 31); and 3) at least part of the interaction between stress and gonadal hormones and their impact on inflammatory processes takes time to develop (15).

In another study involving the knee joint neurogenic inflammation model, sound stress augmented PE in Gx male rats (15). The greater resting PE observed in males appeared to reflect a stress effect may have contributed to the elevated TMJ PE levels observed in males in the present study. In contrast to the influence of sound stress on males, sound stress suppressed PE in the knee joint of females. This suppression was reversed by gonadectomy (15). Thus stress effects may have also contributed to the observed interaction between gonadal hormones and TMJ inflammation in females (i.e., see Fig. 3).

Chronic estrogen replacement was used in the present study to characterize the influence of estrogen on TMJ PE, a measure of one cardinal sign of inflammation: swelling. However, because there are time-dependent influences of estrogen and other hormones associated with the ovulatory cycle, that may be manifest with fluctuating levels of gonadal hormones and because estrogen may have a differential impact on each of the five cardinal signs of inflammation, results of the present study may be relatively specific to the experimental paradigm used. For example, activity in primary afferent neurons is a correlate of a second cardinal sign of inflammation (pain), and there is evidence that estrogen influences primary afferent activity. The excitability of afferents innervating reproductive organs appear to change throughout the estrus cycle (5). However, estrus cycle-dependent changes in the excitability of afferents are not observed in all populations of afferents, in particular, those innervating the TMJ (6). Importantly, there is evidence that both endogenous circulating levels of estrogen (6) and chronic estrogen replacement (12) are sufficient to mediate a persistent increase in TMJ afferent excitability, which may contribute to increased TMD pain (29). Estrogen has also been shown to regulate neuropeptide expression in primary afferents, and neuropeptide release from peripheral terminals may contribute to inflammation-induced changes in blood flow (heat and redness), as well as plasma extravasation (swelling). For example, there is evidence that a transient increase in estrogen is associated with an increase in neuropeptide expression, whereas chronic estrogen treatment decreases these peptides in primary afferents (13, 24, 25). The influence of estrogen on primary afferent excitability and neuropeptide expression provide a clear example of how estrogen may differentially influence the cardinal signs of inflammation in the TMJ; chronic estrogen would contribute to an increase in pain, reflecting in part an increase in afferent excitability while a chronic estrogen-induced suppression of neuropeptides expression would result in a relatively smaller inflammation-associated increases in redness, heat, and swelling.
The estrogen replacement protocol used in Gx+E rats resulted in plasma estrogen levels that were greater than those in intact female rats. Because intact female rats were not staged according to the estrous cycle, plasma estrogen in these rats is expected to be an average of estrogen levels at all stages of the cycle. The hormone replacement protocol used was designed to produce nonfluctuating plasma estrogen levels consistent with those observed in proestrous rats. Although there are advantages and disadvantages to this method of estrogen replacement, the reasons for choosing this paradigm were threefold: 1) to eliminate temporal effects associated with cycling or peak deliveries of estrogen; 2) to achieve an estrogen level that would maximize the ability to detect an estrogen-induced effect; and 3) to obtain results that could be compared with our previously published electrophysiological results (12). However, it is important to note that the difference in estrogen levels between intact and Gx+E rats may contribute to the difference in TMJ PE observed 72 h after CFA injection (Fig. 4).

Why should an estrogen-mediated suppression of PE be associated with an increase in TMJ afferent excitability and increased TMD pain? Although PE is commonly used to quantify inflammation, evidence from rat and human studies suggests that a decrease in PE during inflammation may be associated with an increase in joint damage and pain. For example, it has been demonstrated in a rat arthritis model that the magnitude of PE in the knee joint is inversely proportional to the severity of radiographic joint injury (10, 27). This inverse relationship may account for the observations that signs of polyarthritis develop more quickly and with a greater severity in female rats (1, 11), whereas testosterone appears to have a protective influence against disease progression in this model (18). Interestingly, results from polyarthritis models suggest that there may be differences in various signs of inflammation during disease development as opposed to when the disease is fully manifest. For example, the composite arthritis score used by Allen and colleagues included joint swelling. Citing evidence that estrogen may depress and testosterone may enhance particulate immune complexes (28), Allen and colleagues argued that a similar mechanism may mediate the sex difference observed in the arthritis model, in which deposition of streptococcal cell wall in reticular spaces is thought to contribute to the development of the disease (1). In a similar model, but with different endpoints, mechanical hyperalgesia developed in female rats significantly earlier than in male rats and at a time when there were no differences between groups in paw thickness (11). Results of the present study suggest that the impact of estrogen may be most deleterious during the development of a chronic inflammatory condition. Importantly, in studies of human TMJ synovial fluid from arthritic TMs, concentrations of substance P (which promotes PE) were negatively correlated with TMJ pain and positively correlated with pressure-pain threshold and tolerance over the TMJ (4). Conversely, concentrations of neuropeptide Y (a vasoconstrictive neuropeptide) were positively correlated with spontaneous pain, restricted mandibular mobility, and anterior open bite (a sign of inflammation-induced bilateral destruction of the TMJ) (2, 3). Thus, in the context of our present results, these observations raise the possibility that estrogen may slow recovery from microdamage associated with normal joint articulation, as well as exacerbate damage associated with overt inflammation of the TMJ.

Of note, the impact of estrogen levels on TMJ inflammation were recently reported (16). In this study, TMJ swelling was assessed 24 h after CFA injection, and in contrast to the results of the present study, estrogen appeared to augment TMJ swelling. Although there are several possibilities that may account for different results in the two studies, we suggest that methodological differences played a significant role. For example, in the previous study, TMJ swelling was assessed with visual inspection by a blinded rater (16). Given that the TMJ is a deep structure, covered by fur, skin, and muscles, the sensitivity of visual inspection of the intact animal is likely to be considerably lower than the use of Evans blue plasma extravasation in the joint. It is also interesting to note that in this previous study, the presence of estrogen was associated with a suppression of both CD16+ cells and TNF-α staining in the TMJ 24 h after CFA injection. Given the proinflammatory actions of CD16+ cells and TNF-α, it is reasonable to predict that decreased levels of these markers would correlate with decreases in plasma extravasation, particularly in light of the ability of TNF-α to induce plasma extravasation (22). Thus the impact of estrogen on inflammation-induced changes CD16+ and TNF-α in the TMJ are consistent with results of the present study.

In summary, we observed that there is a sex difference in both resting and inflammation-induced increases in TMJ PE that appeared to reflect a testosterone-mediated facilitation of PE in males and estrogen-mediated suppression of PE in females. Lower PE in females may lead to an increase in joint damage (10, 27). Furthermore, previous observations indicating that estrogen increases the excitability of TMJ afferents (12) suggest that the presence of estrogen may also be associated with an increase in TMJ pain. These observations may help explain both the higher prevalence and severity of TMD pain in females than in males.

ACKNOWLEDGMENTS

We acknowledge Dr. Anne Murphy for helpful discussions regarding the design of the experiments employed.
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GRANTS

This work was supported by National Institutes of Health Grant NS41384.

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