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ESTROGEN AND INFLAMMATION INCREASE THE EXCITABILITY OF RAT TEMPOROMANDIBULAR JOINT AFFERENT NEURONS

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Abstract

Several painful conditions, including temporomandibular disorders (TMD), are more prevalent and more severe in women than in men. Although the physiological basis for this sex difference remains to be determined, it is likely that estrogen is an underlying factor. The present study was performed in order to test the hypotheses that estrogen increases the excitability of rat temporomandibular joint (TMJ) afferents and exacerbates the inflammation-induced sensitization of these sensory neurons. Retrogradely-labeled TMJ neurons from ovariectomized rats and ovariectomized rats receiving chronic estrogen replacement were studied using whole cell patch clamp techniques three days after injecting the TMJ with either saline or Complete Freund's Adjuvant to induce inflammation. Excitability was assessed with depolarizing current injection in order to determine action potential threshold, rheobase and the response to suprathreshold stimuli. Spontaneous activity was also assessed. Both inflammation and estrogen increased the excitability of TMJ neurons as reflected by decreases in action potential threshold and rheobase and increases in the incidence of spontaneous activity. The effects were additive, with neurons from rats receiving both estrogen and inflammation being the most excitable. The increases in excitability were associated with changes in passive properties and action potential waveform, suggesting that estrogen and inflammation affect the expression and/or properties of ion channels in TMJ neurons. Importantly, the influence of estrogen on both baseline and inflammation-induced changes in TMJ neuronal excitability may help explain the profound sex difference observed in TMD, as well as suggest a novel target for the treatment of this pain condition.

Keywords

temporomandibular joint disorder; pain; nociceptor; patch clamp; trigeminal ganglia

INTRODUCTION

Temporomandibular disorders (TMD) are a collection of common conditions that affect the temporomandibular joint (TMJ) and/or the muscles of mastication. TMD is 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 (Carlsson 1999). Furthermore, women who take exogenous estrogens have an increased risk of referral for treatment of TMD pain (LeResche et al. 1997). These observations suggest a potential role for sex hormones, particularly estrogen, in the pathophysiology of TMD pain. However, little is known about the mechanisms of TMD pain or the mechanisms by which estrogen contributes to this pain.

TMD pain may reflect activity in primary afferent neurons innervating the TMJ. Sensory innervation of the TMJ is primarily provided by afferents with cell bodies in the trigeminal ganglion (TG) (Casatti et al. 1999; Uddman et al. 1998; Widenfalk and Wiberg 1990). TG neurons that innervate the rat TMJ give rise to thinly myelinated and unmyelinated fibers that terminate as free nerve endings (Kido et al. 1995). Activity in TMJ afferents may contribute to TMD pain by providing direct nociceptive input into the central nervous system and by contributing to central sensitization (Sessle 1999). Activity in TMJ afferents also facilitates reflex contraction of the masticatory muscles, a phenomenon which is believed to contribute to TMD pain (Cairns et al. 1998; Yu et al. 1995).

The present study was designed to investigate the effects of chronic estrogen on trigeminal primary afferent neurons innervating the TMJ. Specifically, we tested the hypotheses that estrogen increases the excitability of TMJ afferents, and that estrogen exacerbates the inflammation-induced sensitization of TMJ afferents. Retrogradely labeled TMJ neurons from ovariectomized (OVX) female and OVX female rats receiving estrogen replacement were studied *in vitro* with whole cell patch clamp techniques. Neurons were obtained from animals 3 days after a TMJ injection of either saline or Complete Freund's Adjuvant (CFA), a compound used to induce persistent inflammation. Both estrogen and inflammation increased the excitability of TG neurons innervating the TMJ, with neurons from rats receiving both estrogen and inflammation being the most excitable.

METHODS

All procedures involving animals were approved by the University of Maryland Dental School Institutional Animal Care and Use Committee. Female Sprague-Dawley rats (Harlan, 150–225 g) were used for all experiments.

Experimental groups

Neurons were studied from 4 groups of ovariectomized rats distributed in a 2×2 design: one arm of the design was $\pm$ estrogen and the other arm was $\pm$ inflammation. The 4 groups and the acronyms used to describe them are as follows: vehicle replacement saline injected (VS); vehicle replacement CFA injected (VC); estrogen replacement saline injected (ES); and estrogen replacement CFA injected (EC). Seven or 8 rats were studied from each experimental group.

Retrograde labeling

Rats were anesthetized with rat cocktail (55 mg/kg ketamine, 5.5 mg/kg xylazine, and 1.1 mg/kg acepromazine). The skin overlying the TMJ was shaved, and the injection target was identified by palpating the zygomatic arch and mandible. A 30-gauge needle was inserted just inferior to the zygoma until it hit the ramus of the mandible; the needle was then turned superiorly until it was stopped by the glenoid fossa of the squamous bone. The retrograde tracer DiI (3 μ l; 17 mg/ml; Molecular Probes, Eugene, OR (Benson et al. 1999)) was slowly injected into the TMJ. The needle was left in place for ~2 minutes before being withdrawn in order to minimize leakage of dye from the injection site.

Ovariectomy surgery

Ovariectomy (OVX) was performed via a lateral approach. A time-release pellet of 17 β -estradiol (0.25 mg/pellet, 21-day release, Innovative Research of America, Sarasota, FL) or corresponding vehicle was inserted subcutaneously between the shoulder blades. Intramuscular injections of buprenorphine (0.03 mg/kg) and penicillin G (10,000 units/kg) were administered post-operatively.

Induction of inflammation

Rats were anesthetized with rat cocktail, and the injection site was identified as described for the retrograde tracer injection. Unilateral inflammation was induced by injecting CFA (50 μ l; 1:1 in saline; Sigma, St. Louis, MO) into the TMJ using a 30-gauge needle. Non-inflamed rats received vehicle injections (50 μ l saline). CFA or saline was injected slowly over a time span of 2 minutes.

Time line of in vivo manipulations

OVX, implantation of estrogen or vehicle pellet, and injection of retrograde tracer occurred in a single surgical session in order to minimize the number times a rat was anesthetized. Fifteen to 18 days following this surgery, rats were injected with saline or CFA to induce inflammation. Three days following induction of inflammation, rats were sacrificed and trigeminal ganglion (TG) neurons were studied.

Immunohistochemistry

TG from 5 additional rats (2 VS, and 3 ES) were used to assess the percentage of TMJ neurons giving rise to myelinated axons. TG were harvested following transcardiac perfusion with 1 $\times$ phosphate buffered saline (PBS) followed by cold fixative (4% paraformaldehyde in PBS). Ganglia were post-fixed for 3 hours in fixative, equilibrated in 30% sucrose, frozen, sectioned at 16 μ m, and thaw-mounted on lysine-coated slides such that adjacent sections on a slide were separated by 48 μ m of tissue. Two slides from each TG were processed for immunohistochemistry.

The monoclonal antibody N52 (Sigma), which recognizes a 200 kD neurofilament protein (NF200) that is enriched in myelinated neurons, was used to distinguish myelinated from unmyelinated neurons (Lawson et al. 1984; Lawson et al. 1993). Tissue was pre-incubated for 30 min at room temperature in PBS containing 5% normal goat serum and 0.03% Triton $\times$ prior to the addition of primary antibody (1:500) and incubation in a humidified chamber at 4 $^{\circ}$ C overnight. Slides were then washed in PBS for 30 minutes, and Cy2-conjugated secondary antibody (1:200) was applied for 2 hours at room temperature. Slides were washed and cover slipped with PBS and glycerol.

Sections were inspected for the presence of DiI-labeled neurons, and 1 or 2 images were obtained from each slide. Images were acquired on a Nikon microscope fitted with a mercury lamp and filters for the detection of Cy2/FITC/DTAF and Cy3/TRITC/DiI. Images were acquired with a Spot CCD camera with Spot acquisition software. The individual color images were superimposed, contrast balanced, and assembled into double montages. The number of neurons positively labeled with N52 was determined with a combination of Photoshop 5.0 photo editing software (Adobe Systems Incorporated) and NIH imaging software (Scion, Fredrick, MD). Images were converted to grayscale and auto-contrasted using Photoshop 5.0 photo editing software. NIH imaging software was used to analyze cell body size and fluorescence intensity. The nadir between modes of the bi-modal distribution for the fluorescence intensity plot of all neurons was used as the cut-off point between neurons

considered N52 positive (N52+) and neurons considered N52 negative (N52-). Each image was analyzed separately.

Dissociation and cell culture

Rats were deeply anesthetized with rat cocktail. Isolated TG neurons were obtained using a protocol similar to that described elsewhere (Eckert et al. 1998). Briefly, rats were decapitated, and TG removed bilaterally and minced in ice-cold MEM-BS composed of: 90% minimal-essential-medium (MEM; Gibco BRL, Gaithersburg, MD), 10% heat-inactivated fetal bovine serum (BS; Atlanta Biologicals, Norcross, GA), and 1000 units/ml each of penicillin and streptomycin (Gibco BRL). TG were bubbled with carbogen (5% CO₂, 95% O₂) for 45 min at 37°C in 5 ml MEM, to which collagenase P (Boehringer Mannheim, Indianapolis, IN) had been added to a final concentration of 0.125%. TG were then incubated 5 min at 37°C in Ca²⁺- and Mg²⁺-free Hanks balanced salt solution (Gibco, BRL) containing 0.25% trypsin (Worthington, Bristol, UK), 0.025% EDTA (Sigma), and 80 µg/ml DNase (Sigma) following trituration with fire polished Pasteur pipettes. Dissociated TG were run through a percoll gradient to separate myelin debris from cells and plated onto glass cover slips that had been previously coated by a solution of 5 µg/ml mouse laminin (Gibco BRL) and 0.1 mg/ml poly-L-ornithine (Sigma). Two hours after plating, neurons were transferred from a humidified incubator at 37°C and 3% CO₂, to HEPES buffered L-15 media and stored at room temperature. Neurons were studied between 3 and 8 hours after removal from the animal.

Electrophysiology

Patch clamp recordings were performed using a HEKA EPC9 (HEKA Electronik). Data were low-pass filtered at 5–10 kHz with a 4-pole Bessel filter and digitally sampled at 25–100 kHz. Patch pipettes (1–3 MΩ) were filled with (mM): 110 K-Methansulphonate, 10 KCl, 5 Na-methansulphonate, 1 CaCl₂, 2 MgCl₂, 11 EGTA, 10 HEPES, 2 Mg-ATP, 1 Li-GTP; pH 7.2 (adjusted with TRIS-base), 310 mOsm (adjusted with sucrose). Bath solution contained (mM): 140 NaCl, 3 KCl, 2.5 CaCl₂, 0.6 MgCl₂, 10 HEPES, 10 glucose; pH 7.4 (adjusted with TRIS-Base), 325 mOsm (adjusted with sucrose). All salts were obtained from Sigma.

After formation of a tight seal (>5 GΩ) and compensation of pipette capacitance with amplifier circuitry, whole cell access was established. Whole cell capacitance and series resistance were compensated with amplifier circuitry. Cell capacitance was determined with five hyperpolarizing pulses (10 ms) from –60 mV to –80 mV. Input resistance was determined by measuring the current evoked by a –20 mV voltage command from –70 mV to –90 mV. The resting membrane potential (RMP) was determined immediately after the switch to current clamp mode. Neurons were observed for 2 minutes in the absence of any applied stimuli to assess for spontaneous activity. If more than 2 spikes were evoked during this period, the neuron was considered spontaneously active. Mean firing frequency was determined by taking the mean of instantaneous firing frequency for the first 10 spontaneous action potentials, as this number was thought to most closely resemble the firing rate prior to establishing whole cell access. To assess properties of the action potential waveform (overshoot, duration, afterhyperpolarization (AHP) magnitude, rate of decay of the AHP, and maximum rates of rise and fall), a single action potential was evoked with depolarizing current injection (4 ms square pulse at threshold intensity). Overshoot was measured beyond 0 mV. Action potential duration was determined at 0 mV. The magnitude of the AHP was measured relative to the RMP. The decay rate constant of the AHP was determined by fitting the AHP with a single exponential equation. The maximum rates of rise and fall were determined by taking the first derivative of the action potential waveform. A 500 ms square-wave depolarizing current injection was used to assess neuronal excitability (action potential threshold, rheobase, and the response to suprathreshold stimuli). Action potential threshold was defined as the maximum depolarization obtained in the absence of an action potential (Figure 1A, D). Rheobase was defined as the

minimum amount of depolarizing current sufficient to evoke an action potential (Figure 1A, D). The response to suprathreshold stimuli was assessed by counting the number of action potentials evoked by depolarizing current injections equal to 2× and 3× rheobase (Figure 1B, C, E, F). Differences in the response to suprathreshold stimuli were assessed in two ways: 1) by comparing the slopes of the stimulus-response function for each neuron, as determined by linear regression, and 2) by comparing the proportion of neurons firing > 1 action potential at 2× and 3× rheobase. Excitability was assessed from the RMP in each neuron.

Subcategorization of neurons

In order to further characterize TMJ neurons, we assessed the presence of isolectin B4 (IB4) binding and capsaicin sensitivity in subpopulations of TMJ neurons. That is, neurons from 5 of 8 VS, 4 of 7 VC, 5 of 7 ES and 6 of 8 EC rats were studied with IB4, and neurons from 4 of 8 VS, 3 of 7 VC, 3 of 7 EC and 4 of 8 EC rats were studied with capsaicin. Neurons were labeled with IB4 with a 10 minute incubation in bath solution containing 10 µg/ml FITC-labeled IB4 (Sigma) prior to recording; labeled neurons (IB4+) were easily distinguished from unlabeled neurons (IB4−) under epifluorescence illumination. Capsaicin sensitivity was assessed after the completion of all other protocols with a 500 µl bolus (via hand held pipette) application of 500 nM capsaicin (Sigma, diluted from a 10 mM stock solution in 100% ethanol). Preliminary experiments indicated that a similar bolus application of vehicle could produce a transient change in membrane potential as large as 20 mV. Therefore, neurons were considered capsaicin sensitive (CAP+) if capsaicin resulted in a depolarization > 20 mV. Neurons were considered unresponsive (CAP−) if capsaicin failed to depolarize the membrane potential > 20 mV. Of note, while many investigators use cell body size as a method to subcategorize sensory neurons, we chose not to subcategorize TMJ neurons by this criteria in the present study for 3 main reasons: First, the correlation between cell body size and axon conduction velocity has been primarily established for cutaneous afferents (Lawson et al. 1993). Second, we are not aware of any data from the TMJ suggesting that such a correlation exists for sensory innervation of this structure. And third, it was recently demonstrated that there is no correlation between cell body size and the putative axon conduction velocity of sensory neurons innervating the rat colon (Gold et al. 2002).

Plasma estrogen determination

Immediately prior to decapitation, 1 ml of blood was collected in a heparinized syringe via the left cardiac ventricle. Blood was stored at 4°C for at least one hour prior to being spun for 10 min at 14,000 rpm. The plasma was removed and stored at −20°C until the estrogen concentrations were determined via radioimmunoassay at a commercial laboratory (Analytics, Charlotte, VA).

Statistical analyses

A two-way ANOVA was used to assess the effects of estrogen and inflammation, as well as potential interactions between these two variables, on most parameters studied. If raw data were not normally distributed, they were converted to ranks, and a two-way ANOVA was performed on ranked data. Data not normally distributed included cell body size and the response to suprathreshold stimuli. If the two-way ANOVA revealed significant interaction effects then post-hoc all pair-wise multiple comparisons were performed using the Holm-Sidak test. The Chi-square test was used to assess differences in the proportion of spontaneously active neurons and the proportion of neurons firing > 1 action potential in response to suprathreshold stimuli. Other tests were applied as needed to make specific statistical comparisons between groups and these tests are indicated when used. Statistical significance was assessed at $p < 0.05$.

RESULTS

Plasma estrogen levels

Technical difficulties precluded accurate measurement of plasma estrogen concentrations from all but 5 of the rats used in this study: 2 VS, 1 VC and 2 EC rats. Of these rats, plasma estrogen concentrations were below the level of detection (~ 2 pg/ml) in all vehicle treated rats. The concentrations in the 2 EC rats were 76.2 and 135.2 pg/ml. In order to rule out the possibility that inflammation influenced plasma estrogen concentration, estrogen concentrations were assessed in a second group of rats treated identically to those used in the present study. Mean plasma estrogen concentrations were 1.4 ± 1.4 (n = 7), 0.1 ± 0.1 (n = 22), 95.7 ± 15.0 (n = 12) and 99.9 ± 17.5 (n = 11) pg/ml for VS, VC, ES and EC groups, respectively. As expected, estrogen had a significant effect on plasma estrogen level ($p < 0.01$, $F = 78.2$). Importantly, however, inflammation did not influence plasma estrogen level.

Retrograde labeling

In the present study, the retrograde tracer DiI was injected into the TMJ in order to identify *in vitro* TG neurons that had innervated the TMJ. Post-mortum inspection of the TMJ tissues showed that the retrograde tracer injections consistently targeted the TMJ, with minimal leakage to surrounding tissues. Labeling intensity was more than sufficient to unambiguously distinguish labeled from unlabeled neurons (Figure 2).

Immunohistochemistry

Because the soma size of TG neurons is on average smaller than that of dorsal root ganglion (DRG) neurons (Potrebic et al. 2003), and there is suggestive evidence that innervation of the rat TMJ is restricted to A δ - and C-fibers (Cairns et al. 2001; Kido et al. 1995; Takeuchi et al. 2001), the fraction of TMJ afferents giving rise to myelinated axons was determined. N52, a monoclonal antibody against a 200 kd neurofilament that is enriched in neurons with myelinated axons, was used to determine the fraction of TMJ neurons with myelinated axons (Lawson et al. 1984; Lawson et al. 1993). The percentage of N52+ neurons among TG was $22.5 \pm 1.9\%$ (n = 5 rats). 20% of TMJ neurons were double labeled with N52. However, all of the double labeled neurons were scored as just above cut-off for a neuron to be considered N52 + (Figure 2), suggesting that these neurons gave rise to thinly myelinated axons. Uncorrected for cell shrinkage associated with fixation, the soma size of N52+ neurons was $32.0 \mu\text{m}$ (29 – 36 μm ; median with 25th – 75th percentiles), while that of the total population of neurons was $27 \mu\text{m}$ (22 – 32 μm) and that of TMJ neurons was $24 \mu\text{m}$ (20 – 29 μm) (Figure 2B). These differences were all statistically significant ($p < 0.01$, One Way ANOVA on ranks (where H = 65.7) followed by Dunn's post hoc test).

Capsaicin sensitivity and IB4 binding

Capsaicin responsiveness was assessed in a subset of TMJ neurons from each experimental group. CAP+ neurons responded to a bolus application of 500 nM capsaicin with a large depolarization that was usually associated with a burst of action potentials (Figure 2C). CAP – neurons demonstrated little change in membrane potential in response to the same stimulus (Figure 2D). Overall, 71% (50/70) of TMJ neurons were responsive to capsaicin. CAP+ TMJ neurons were significantly ($p < 0.05$, $T=487$, Mann-Whitney Rank Sum Test) smaller than CAP– TMJ neurons: cell capacitances were 34.5 pF (29 – 50 pF; median and 25th – 75th percentiles) and 64.8 pF (32 – 81 pF) for CAP+ and CAP– TMJ neurons from vehicle treated rats, respectively. There was no statistically significant difference ($p > 0.05$, $\chi^2 = 0.29$) between groups with respect to the proportion of CAP+ neurons: 13/19 VS, 9/12 VC, 17/23 ES and 11/16 EC neurons were CAP+. Nor was there a difference between groups ($p > 0.05$, One Way ANOVA: $F = 0.17$) with respect to the maximal capsaicin evoked changes in membrane

potential which were 52 ± 4 mV, 53 ± 5 mV, 51 ± 4 mV and 55 ± 4 mV for VS, VC, ES and EC neurons, respectively.

IB4 staining was also assessed in a subset of TMJ neurons from each of the experimental groups studied. Overall, 29% (42/147) of TMJ neurons were IB4+. There was no statistically significant ($p > 0.05$, $T=1027$, Mann-Whitney Rank Sum Test) difference between IB4+ and IB4- TMJ neurons with respect to cell body size: cell capacitances were 35.9 pF (32 – 62 pF) and 40.2 pF (30 – 63 pF) for IB4+ and IB4- TMJ neurons from vehicle treated rats, respectively. There was also no statistically significant difference ($p > 0.05$, $\chi^2 = 1.49$) between groups with respect to the proportion of IB4+ TMJ neurons: 8/28 VS, 9/27 VC, 12/36 ES and 13/56 EC neurons were IB4+. Because only a subset of TMJ neurons was studied with IB4, it was possible to determine whether IB4 staining influenced neuronal excitability. The properties of IB4 treated neurons (both IB4+ and IB4-) were compared to those of untreated TMJ neurons within each experimental group. Importantly, there were no statistically significant differences between IB4 treated and untreated neurons (data not shown).

There was no statistically significant difference between CAP+ and IB4+ TMJ neurons with respect to cell body size. Nor was there a statistically significant difference in the percentage of CAP+ (29%, 18/62) and CAP- (21%, 4/19) TMJ neurons that were IB4+.

Spontaneous activity (SA)

A neuron was considered spontaneously active if more than two action potentials were fired during a two-minute observation period immediately after establishing whole cell access. Examples of spontaneous activity (SA) observed in neurons from ES and EC groups are shown in Figure 3. The majority of SA neurons demonstrated irregular activity at a relatively low frequency (Figure 3A, Table 1). However, some neurons, particularly those from EC animals, displayed regular activity at a relatively high frequency (Figure 3B, Table 1). There was a statistically significant difference between groups with respect to the percentage of SA neurons ($p = 0.01$, $\chi^2 = 10.86$, Table 1). There was no difference between groups with respect to mean firing frequency of spontaneously active neurons (Table 1) or the total number of spikes fired within the 2 minute period, which were 78 ± 73 , 76 ± 52 , 128 ± 35 and 133 ± 32 in neurons from VS, VC, ES and EC rats, respectively. There was also no difference between groups with respect to the cell body capacitance of SA neurons (Table 1). As a group, SA neurons (-47.3 ± 0.9 mV; $n = 44$) were more depolarized than non-SA neurons (-55.7 ± 0.4 mV; $n = 180$, $p < 0.01$, $t = 8.42$). However, there was a statistically significant ($p < 0.05$, $F = 7.78$) interaction between estrogen and inflammation with respect to RMP (Table 1). Consistent with the suggestion that the majority of SA neurons were nociceptive, all of the SA neurons (23/23, including 2 VS, 2 VC, 11 ES and 8 EC) challenged with capsaicin were CAP+. As with the total population of TMJ afferents, the majority of SA neurons assessed for IB4 binding were IB4- (29/33). Of note, the percentage (12%) of IB4+ SA neurons was significantly ($p = 0.01$, $\chi^2 = 6.52$) smaller than the percentage (34.5% (38/110) of IB4+ non-SA neurons.

Estrogen-induced effects

As described in Methods, action potential threshold, rheobase, and the response to suprathreshold stimuli were used to assess excitability. Excitability was measured from the RMP of each neuron. Since SA neurons were active at rest by definition, these neurons were excluded from subsequent analyses. Chronic estrogen treatment resulted in a significant decrease in both action potential threshold ($p < 0.05$, $F=3.95$; Figure 4A) and rheobase ($p < 0.01$, $F=11.8$; Figure 4B). There was no statistically significant main effect due to estrogen on the slope of the stimulus response function (Figure 4C).

Estrogen appeared to have a differential influence on the excitability of subpopulations of TMJ afferents defined by capsaicin sensitivity and IB4 binding. In contrast to all non-SA neurons, estrogen had no statistically significant influence on the excitability of either CAP+ or IB4+ TMJ neurons (Table 2); although the apparent absence of an influence on CAP+ neurons may be due to the fact that spontaneously active neurons were excluded from this analysis. Importantly, the direction of the impact of estrogen on rheobase in both CAP+ and IB4+ neurons was the same as in the population as a whole. There was, however, a statistically significant influence of estrogen on the excitability of both CAP- and IB4- neurons (Table 2) with a significant decrease in rheobase in CAP- neurons and a decrease in both rheobase and threshold in IB4- neurons.

It is noteworthy that there were differences between subpopulations of TMJ neurons defined by IB4 binding and capsaicin sensitivity in terms of both baseline excitability and the response to estrogen. In VS rats, CAP+ neurons were the most excitable in terms of action potential threshold ($p < 0.05$, One way ANOVA, $F = 2.94$, Holm-Sidak post hoc test), rheobase ($p = 0.01$, $F = 4.19$) and normalized rheobase ($p < 0.05$, Kruskal Wallis one way ANOVA, $H = 9.02$), and CAP- neurons from VS treated animals were the least excitable. In contrast, estrogen was associated with the greatest increase in excitability in CAP- neurons (Table 2). And while IB4-, IB4+ and CAP+ subpopulations of TMJ neurons were similar to that of all non-SA neurons with respect to the influence of estrogen (Table 2), significant effects were only detected in the IB4- and CAP- subpopulations.

To begin to address potential changes in ionic currents that could be responsible for the estrogen-induced increase in excitability, we compared the passive properties of each group of neurons. Estrogen did not significantly impact membrane capacitance or input resistance (Table 3). However, there was a significant influence of estrogen on RMP such that the RMP of neurons from estrogen treated rats was more depolarized than that of vehicle treated rats ($p < 0.05$, $F = 4.9$; Table 3). There was a differential influence of estrogen on subpopulations of TMJ neurons defined by IB4 binding and capsaicin responsiveness such that estrogen was associated with a significant depolarization of RMP in both IB4+ ($p < 0.05$, $F = 5.58$) and IB4- ($p < 0.01$, $F = 12.92$), but not in CAP+ or CAP- neurons. There was no significant influence of estrogen on input resistance in these subpopulations of TMJ neurons.

To further investigate the ionic mechanisms underlying the influence of estrogen on excitability, the action potential waveform of each neuron was analyzed as described in Methods. Importantly, there was no significant influence of estrogen on any aspect of the action potential waveform in all non-SA neurons (Table 4). However, estrogen was associated with a decrease in action potential duration ($p < 0.05$, $F = 4.67$) and increase in the rate of action potential rise ($p < 0.05$, $F = 4.08$) in IB4+ neurons and an increase in the rate of action potential rise in CAP+ neurons ($p = 0.03$, $F = 4.54$) (data not shown).

Inflammation-induced effects

CFA-induced inflammation of the TMJ resulted in an increase in the excitability of TMJ neurons. This was manifest as a decrease in both action potential threshold ($p < 0.01$, $F = 12.1$; Figure 4A) and rheobase ($p < 0.01$, $F = 10.7$; Figure 4B). These changes were observed in the absence of a significant change in the slope of the stimulus-response function (Figure 4C).

As with estrogen, inflammation appeared to have a differential influence on the excitability of subpopulations of TMJ afferents defined by capsaicin sensitivity and IB4 binding. That is, there was no significant influence of inflammation on the excitability of CAP- neurons, and an inflammation-induced decrease in rheobase, but not threshold, was detected in IB4+ and IB4- neurons (Table 2). However, significant decreases in both action potential threshold and rheobase were observed in CAP+ neurons (Table 2).

Two passive properties assessed in TMJ neurons were significantly influenced by inflammation. The most prominent change was a significant ($p < 0.01$, $F=7.63$) inflammation-induced increase in cell body capacitance (Table 3). This change is illustrated by a clear rightward shift in the peak of the cell body capacitance histogram for neurons from inflamed rats compared to that of saline treated controls (Figure 5). Inflammation was also associated with a significant ($p = 0.03$, $F=5.10$) effect on input resistance of all non-SA neurons such that the input resistance was lower in neurons from inflamed rats (Table 3). Similar changes were observed in CAP+ neurons, although the decrease in input resistance did not reach statistical significance ($p = 0.08$). While not detected in all non-SA neurons, inflammation was associated with a significant depolarization of IB4+ ($p < 0.05$, $F=4.66$), and CAP- ($p < 0.05$, $F=4.68$) TMJ neurons (data not shown). There was no statistically significant effect of inflammation on any component of the action potential waveform that was assessed in all non-SA neurons (Table 4) nor in subpopulations of TMJ neurons defined by IB4 binding and capsaicin sensitivity (data not shown).

Interactions between estrogen and inflammation

Two-way ANOVA of estrogen- and inflammation-induced changes in the excitability of TMJ neurons indicated that there was no significant interaction between estrogen and inflammation with respect to the excitability of TMJ neurons when analyzed as all non-SA neurons or as subpopulations of CAP+, CAP- or IB4+ ($p > 0.05$, Figure 4, Table 2). However, there was a significant interaction between estrogen and inflammation in IB4- neurons. That is, inflammation increased the excitability of neurons from vehicle treated rats (with decreases in both action potential threshold and rheobase), but inflammation decreased excitability in estrogen treated rats (with increases in both action potential threshold and rheobase, Table 2).

Similar analysis of passive membrane properties revealed a significant ($p < 0.01$, $F=10.22$) interaction between estrogen and inflammation in their influence on RMP (Table 4). That is, inflammation was associated with RMP depolarization within the vehicle treated group and hyperpolarization within the estrogen treated group. Estrogen was also associated with RMP depolarization within the vehicle treated group. Similar results were obtained in IB4+ ($p < 0.05$, $F=4.66$) and IB4- ($p < 0.05$, $F=6.16$) TMJ neurons (data not shown). Analysis of the properties of the action potential waveform in all non-SA neurons revealed a significant interaction between estrogen and inflammation on the rate of action potential fall (Table 5). In the absence of estrogen, inflammation was associated with a decrease in the rate of action potential fall, while in the presence of estrogen, inflammation was associated with an increase in the rate of fall (Table 4). There were no statistically significant differences among experimental groups with respect to properties of the action potential waveform within subpopulations of TMJ neurons defined by IB4 binding and capsaicin responsiveness.

The overall response of TMJ neurons to suprathreshold stimuli was relatively small. This was particularly striking for neurons taken from CFA treated animals in light of previous results demonstrating dramatic inflammation-induced increases in the response to suprathreshold stimuli in neurons innervating other structures (Yoshimura and de Groat 1999). Nevertheless, further analysis of these data revealed two populations of neurons that could be classified as firing a single action potential or >1 action potential in response to suprathreshold stimuli (Table 5). There was a statistically significant difference between the 4 groups in the percentage of neurons firing > 1 action potential at both $2\times$ ($p = 0.04$, $\chi^2=8.10$) and $3\times$ rheobase ($p<0.01$, $\chi^2=12.02$), with the greatest percentage of neurons from EC rats firing >1 action potential. When individual groups were compared, the percentage of EC neurons firing > 1 action potential at $2\times$ rheobase was significantly greater than that of VS ($p = 0.04$, $\chi^2=4.01$), and VC ($p = 0.02$, $\chi^2=5.81$) neurons. The percentage of EC neurons firing > 1 action potential at $3\times$

rheobase was significantly greater than that of each of the 3 other groups: VS ($p < 0.01$, $\chi^2 = 10.48$), VC ($p = 0.03$, $\chi^2 = 4.44$), and ES ($p = 0.04$, $\chi^2 = 4.24$).

DISCUSSION

The present study was designed to test the hypotheses that chronic estrogen treatment increases the excitability of primary afferent neurons innervating the TMJ, and that estrogen exacerbates the inflammation-induced sensitization of these neurons. Our primary findings were: 1) Chronic estrogen was associated with decreases in action potential threshold and rheobase in TMJ neurons; 2) Persistent inflammation was associated with decreases in action potential threshold and rheobase in TMJ neurons; and 3) TMJ neurons from estrogen and CFA treated rats were the most excitable.

General observations

The absence of intense N52 staining of TMJ neurons and only light N52 staining in the minority of these neurons suggests that the TMJ is innervated by afferents with unmyelinated and thinly myelinated (A δ - and C-fiber) axons. That all TMJ afferents had an inflexion on the falling phase of the action potential suggests that these neurons gave rise to high threshold receptors (Ritter and Mendell 1992). Furthermore, the observation that the majority of TMJ afferents were responsive to capsaicin suggests that these afferents are nociceptive. These observations are consistent with previous electrophysiological and anatomical data. For example, results from an electrophysiological study using a mechanical search stimulus indicate that the TMJ is innervated by high threshold afferents with slowly (A δ - and C-fiber) conducting axons (Cairns et al. 2001). Similar results were obtained in a second electrophysiological study employing high threshold mechanical and thermal stimuli, as well as noxious chemical (bradykinin) stimuli (Takeuchi et al. 2001); although in this latter study, the investigators focused on A δ - and C-fibers and therefore ignored the presence of units with more rapidly conducting axons. Importantly, ultra-structural analysis of TMJ innervation in the rat indicates that afferents arising from the TG terminate in free nerve endings (Kido et al. 1995), which is a property characteristic of nociceptive afferents (Kruger et al. 2003; Kruger et al. 1981).

The excitability of isolated neurons *in vitro* was assessed with depolarizing current injection, 3 to 8 hours after harvesting neurons from the animal. Results obtained with this approach have several important implications. First, because the stimulus presumably bypasses transduction processes, estrogen- and inflammation-induced increases in excitability must reflect intrinsic differences in the properties of ion channels present in the plasma membrane. Thus, differences in excitability between groups of neurons should be manifest *in vivo* regardless of the stimulus modality to which the neurons are exposed. Second, the time delay between tissue harvest and electrophysiological recording suggests increases in excitability reflect transcriptional regulation of ion channel(s) expression. Both estrogen and inflammatory mediators have been shown to acutely modulate the activity of several ion channels. Indeed, there is evidence that estrogen inhibits L-type voltage gated Ca²⁺ channels in rat dorsal root ganglion neurons (Chaban et al. 2003). Estrogen may also increase the activity of Ca²⁺-dependent K⁺ channels (Valverde et al. 1999) which are also present in sensory neurons (Scholz et al. 1998). Similarly, inflammatory mediators have been shown to acutely modulate voltage-gated Na⁺, Ca²⁺ and K⁺ channels as well as Ca²⁺-dependent K⁺ channels (Gold 2001). Therefore, the possibility that estrogen- and inflammation-induced changes in excitability reflect a long-lasting change in the biophysical properties of an ion channel(s) resulting from post-translational modifications cannot be excluded. However, the fact that estrogen-induced changes in ion channel function are readily reversible (Chaban et al. 2003), suggests this possibility is unlikely to account for the influence of estrogen in the present study. Furthermore, that the properties of neurons studied 3–4 hours after dissociation were indistinguishable from those of neurons

studied 7–8 hours after dissociation (data not shown) argues against a slow wash-out of acute estrogen-induced changes in excitability. Third, it is likely that the inflammation-induced increase in excitability will be considerably larger *in vivo* than observed in the present study. This suggestion is based on the fact that persistent inflammation will be associated with elevated levels of inflammatory mediators. Many of these compounds act directly on primary afferent neurons to increase excitability via post-translational modification of both transducers and ion channels (Levine and Reichling 1999) present in peripheral terminals. Thus, if the changes we observed in the cell body are present in the peripheral terminals, they will be augmented by the direct actions of inflammatory mediators. And fourth, while it is generally assumed that changes observed at the soma will be present in afferent terminals (Gold 2001), even if this assumption is false, observations from the present study may still be physiologically relevant. That is, an increase in the excitability of the afferent soma *in vivo* may still contribute to increased pain associated with tissue injury. Such changes have been well documented following nerve injury (Kajander et al. 1992; Liu et al. 2000), and may also contribute to pain associated with inflammatory conditions (Djouhri and Lawson 2001).

Estrogen and inflammation were both associated with decreases in action potential threshold and the amount of current necessary to evoke an action potential. That a concomitant increase in the response to suprathreshold stimuli was not observed does not indicate that the change in excitability was somehow incomplete. Rather, these observations suggest that the increase in excitability of TMJ afferents is distinct from that observed in other systems. For example, an inflammation-induced parallel shift in the stimulus response function as reflected by a decrease in threshold and an increase in the response to suprathreshold stimuli has been observed in colonic neurons (Su et al. 1997). In contrast, an inflammation-induced change in slope of the stimulus response function was observed in cutaneous nociceptors (Andrew and Greenspan 1999a). The “mode” of sensitization observed in the present study which was associated with a decrease in threshold with no change in the suprathreshold response is similar to that observed in response acute application of the inflammatory mediator serotonin to sensory neurons *in vitro* (Cardenas et al. 2001).

Data from single unit studies from intact preparations suggests that the incidence of resting or spontaneous activity in naïve tissue varies as a function of the tissue studied. For example, cutaneous afferents exhibit little or no resting activity (Andrew and Greenspan 1999b), while there appears to be considerable resting activity in muscle (Berberich et al. 1988) and knee joint (Schaible and Schmidt 1985). In all three of these preparations, persistent inflammation is associated with a dramatic increase in both the incidence and frequency of spontaneous activity (Andrew and Greenspan 1999a; Berberich et al. 1988; Schaible and Schmidt 1985). Similar results have recently been described in an *in vitro* TMJ preparation (Takeuchi et al. 2004). Results from the present study suggest that resting activity in deeper structures like muscle and joint as well as the increase in activity following inflammation, reflects, at least in part, intrinsic properties of the afferents innervating these structures. Interestingly, in the present study chronic estrogen treatment alone was associated with a dramatic increase in spontaneous activity in TMJ neurons. While the low frequency of activity associated with the spontaneous activity was unlikely to be overtly painful, as suggested by the normal feeding and grooming of ES rats, it may serve to lower the threshold for the manifestation of overt pain arising from the TMJ.

Estrogen effects

The chronic estrogen-induced increase in excitability was associated with membrane depolarization. However, estrogen had little effect on the action potential waveform. These results suggest that estrogen primarily influences the excitability of TMJ neurons via a change in a resting membrane conductance. This is likely to be an increase in a depolarizing

conductance rather than a decrease in a hyperpolarizing conductance because estrogen tended to decrease input resistance (although the effect was not statistically significant).

Chronic estrogen not only increased the incidence of spontaneous activity, but decreased action potential threshold and rheobase. This increase in excitability suggests that in the presence of high estrogen, rats should be more responsive to noxious stimulation of the TMJ. This suggestion is consistent with previous experimental data indicating that there is a sex difference and estrogen dependence to TMJ pain evoked with chemical stimuli. For example, female rats exhibited a greater response than males in primary afferent activity and in the reflex muscle activity that was evoked upon injection of glutamate into the TMJ (Cairns et al. 2001; Cairns et al. 2002). Furthermore, ovariectomy of female rats decreased the glutamate-evoked muscle activity, but estrogen replacement increased it (Cairns et al. 2002). In another model, injection of the small fiber irritant mustard oil into the rat TMJ produces neuronal activation in the spinal trigeminal nucleus. This neuronal activation, as measured by fos-immunoreactivity, was greater in proestrous females (with high serum estrogen levels) than in males or diestrous females (with low serum estrogen levels) (Bereiter 2001). Results from the present study suggest that these previous observations may reflect, at least in part, estrogen-induced changes in the intrinsic excitability of TMJ afferents.

While chronic estrogen treatment was used in the present study, it is important to point out that temporal aspects of estrogen treatment may have important consequences, particularly with respect to nociceptive processing. For example, the response properties of afferents innervating the reproductive organs of the rat change as a function of stage of the estrous cycle (Robbins et al. 1992). There is also data, such as that described above from the TMJ (Cairns et al. 2002) as well as from studies of the rat hindpaw (Dina et al. 2001; Joseph et al. 2003) suggesting afferent properties are influenced by the presence of estrogen, but that these properties persist in the face of fluctuations in estrogen levels that occur over the rat estrous cycle. Thus, activational influences of estrogen on these populations of afferents appear to outlast the time course of the rat estrous cycle. Recent evidence from a human psychophysical study suggests that rapid changes in estrogen levels that occur across the menstrual cycle may also influence pain, particularly temporomandibular pain (LeResche et al. 2003). Thus, the change in estrogen, rather than the absolute amount of estrogen, may be important for some physiological processes. Finally, it is also worth noting that the duration of estrogen treatment may also influence afferent properties. For example, in a normal cycling rat, estrogen receptor (ER) α and ER β expression levels are both higher during proestrus (high estrogen levels) than during metestrus (low estrogen levels), while long term estrogen treatment results in a decrease in ER α levels and an increase in ER β levels (Taleghany et al. 1999). Thus, time dependent changes in the influence of estrogen on the excitability of TMJ afferents are an important issue that should be pursued in the future.

Systemically administered estrogen could act at several different sites to produce the changes in ion channels that we have detected. The simplest explanation is a direct effect mediated by the classic steroid hormone mechanism, where the estrogen/receptor complex influences gene transcription. Although we are unaware of any reports indicating estrogen receptor α (ER α) or β (ER β) are either present or absent in TG neurons, these receptors have been localized in primary afferents in the dorsal root ganglia (Sohrabji et al. 1994; Taleghany et al. 1999). A second possible mechanism could involve a membrane-bound receptor coupled to a signal transduction pathway (Toran-Allerand et al. 2002; Toran-Allerand et al. 1999). The effects of estrogen on TMJ afferents also may be indirect, even in the absence of overt inflammation. For example, recent evidence suggests that estrogen receptors are present in a number of cell types lining the TMJ (Yamada et al. 2003). Furthermore, estrogen increases levels of nerve growth factor (NGF) in some peripheral tissues (Bjorling et al. 2002), and estrogen modulates the levels of the NGF receptor, TrkA, in primary afferent neurons (Lanlua et al. 2001; Liuzzi

et al. 1999; Sohrabji et al. 1994). These observations with NGF and TrkA receptors are particularly intriguing because NGF produces several pro-nociceptive changes in primary afferents (Woolf 1996), and has been shown to modulate expression of voltage-gated ion channels in models of peripheral nerve injury (Black et al. 1997). Finally, an indirect action via the central nervous system is also possible. Estrogen receptors are found throughout the CNS, including a number of regions known to be involved in the modulation of nociceptive information. It should also be noted that the loss of estrogen following ovariectomy may result in an increase in the excitability of TMJ afferents secondary to an increase in hypothalamic and pituitary hormones in the absence of feedback inhibitory control. This effect of ovariectomy is unlikely, however, given that the excitability of TMJ neurons from OVX rats is similar to that of TMJ neurons from intact male rats (data not shown).

Inflammation-induced effects

Persistent inflammation *in vivo* was associated with an increase in the excitability of TMJ neurons *in vitro*. Coincident with the increase in excitability was a decrease in input resistance and an increase in capacitance. As with the estrogen-induced increase in excitability, the inflammation-induced decrease in input resistance is consistent with an increase in a depolarizing cationic conductance.

The observation that persistent inflammation of a peripheral structure results in an increase in excitability of sensory neurons that is detectable in the cell body *in vitro* is consistent with results from other investigators using inflammatory models in different tissues (Bielefeldt et al. 2002a; Dang et al. 2003; Moore et al. 2002; Yoshimura and de Groat 1999). Interestingly, both the pattern of inflammation-induced excitability changes and the associated changes in passive properties, properties of the action potential waveform, or specific ion channels varies from study to study. For example, cyclophosphamide-induced bladder inflammation results in a profound increase in the number of action potentials fired in response to a prolonged stimulus that appears to reflect a decrease in an A-type voltage-gated K^+ current (Yoshimura and de Groat 1999). In contrast, a trinitrobenzene sulfonic acid (TNBS) model of ileitis results in a decrease in action potential threshold that is associated with no change in membrane potential and an increase in membrane resistance and rate of action potential rise (Moore et al. 2002). The changes in the TNBS model appear to reflect a decrease in both an A-type and a sustained K^+ current as well as an increase in a voltage-gated Na^+ current (Stewart et al. 2003). A third model of visceral inflammation involving acetic acid-induced gastric ulceration (Bielefeldt et al. 2002b) appears to reflect a leftward shift in a voltage-gated Na^+ current and decreases in an A-type K^+ current with no change in sustained K^+ current (Bielefeldt et al. 2002b; Dang et al. 2003; Moore et al. 2002). It is possible that differences between these studies as well as the present study reflect differences in experimental methods (i.e., type of inflammation, timing between induction of inflammation and study of neurons, sex of animals). However, another intriguing possibility is that the response to inflammation of specific subpopulations of sensory neurons defined by target of innervation may be unique. Consistent with this possibility, it was recently reported that there are marked differences between colonic and cutaneous afferents *in vitro* in response to acute application of the inflammatory mediator PGE_2 (Gold and Traub 2004).

The inflammation-induced increase in cell body size observed in TMJ afferents is similar to that observed in both bladder (Yoshimura and de Groat 1999) and gastric (Bielefeldt et al. 2002a) afferents. That such a change has been observed in afferents innervating 3 different tissue types inflamed with different stimuli suggests that an increase in cell body capacitance may be a general response to inflammatory tissue injury. The basis for such an increase is unclear, but may reflect an increase in membrane production that would be associated with the sprouting of peripheral terminals *in vivo*.

TRPV1, the capsaicin receptor, appears to be critical for the expression of inflammatory hyperalgesia (Caterina and Julius 2001). The receptor is acutely modulated by a number of inflammatory mediators and is upregulated in the presence of persistent inflammation (Amaya et al. 2003; Carlton and Coggeshall 2001; Ji et al. 2002). Furthermore, there is evidence that in the presence of inflammation, there is an increase in the distribution of TRPV1 among DRG neurons (Amaya et al. 2003). In contrast, there was no detectable inflammation-induced increase in the number of capsaicin responsive TMJ neurons observed in the present study. The basis for this apparent discrepancy is unclear but may reflect differences in the site of inflammation (joint versus hindpaw), the ganglia influenced (DRG versus TG), or the fact that the majority of TMJ neurons were capsaicin sensitive in non-inflamed rats. That target of innervation/inflammation may be the critical factor is suggested by the observation that antigen-induced arthritis of the knee joint is not associated with an increase in the proportion of joint afferents expressing TRPV1 (Bar et al. 2004). Because capsaicin was used in the present study as a means of assessing the extent to which TMJ neurons exhibited properties of putative nociceptive afferents, capsaicin evoked responses were not assessed in a manner that would enable the detection of an inflammation-induced increase in the magnitude of the capsaicin evoked response. That is, in current clamp, capsaicin will ultimately drive the membrane potential of capsaicin responsive neurons to that of the equilibrium potential for TRPV1 ($\sim +10$ mV under the ionic conditions employed in the present study) as was observed in the majority of cases in the present study. Thus, the possibility that inflammation of the TMJ results in an up-regulation of TRPV1 in TMJ afferents should be explored in more detail in the future.

Interactions between estrogen and inflammation

Chronic estrogen and persistent inflammation were both associated with an increase in the excitability of TMJ neurons. While neurons from EC rats were the most excitable across all measures of excitability, including spontaneous activity, action potential threshold, rheobase and the response to suprathreshold stimuli, the difference between neurons from ES and EC rats was not as great as might have been expected, even if the influence of estrogen and inflammation were only additive. There are at least two likely explanations for this result. First, there appeared to be an influence of a floor effect, in that there are limits constraining the maximum possible decrease obtainable for both threshold and rheobase. Second, while there appeared to be differences in the underlying mechanisms of estrogen- and inflammation-induced increases in excitability, there also appeared to be common mechanisms as well. An example of the latter may be the conductance driving membrane depolarization and a decrease in membrane resistance. In contrast, the influence of CFA on the rate of action potential fall was dependent on the presence of estrogen. If, for example, the inflammation-induced decrease in the rate of action potential fall observed in neurons from vehicle treated rats reflects a decrease in A-type K^+ current described above in other inflammation models (Bielefeldt et al. 2002b; Dang et al. 2003; Moore et al. 2002), then modulation of another ionic current would have to account for the inflammation-induced increase in the rate of action potential fall observed in neurons from estrogen treated rats. That distinct mechanisms underlie the influences of estrogen and inflammation is also suggested by the observation that these two manipulations had different effects in different subpopulations of TMJ neurons defined by IB4 binding and capsaicin sensitivity. This was particularly true in IB4 $^-$ neurons where the particular response to inflammation was clearly dependent on the presence of estrogen.

Summary

We observed estrogen- and inflammation-induced increases in the excitability of TMJ afferents *in vitro*. These changes in excitability reflect changes in the intrinsic properties of TMJ afferents and are likely to be augmented by the presence of inflammatory mediators *in vivo*. Importantly, the combined influence of estrogen and inflammation may contribute to the profound sex difference observed in the frequency, duration and severity of TMD pain in humans.

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Figure 1. Excitability of trigeminal ganglion neurons innervating the TMJ

Excitability was measured in DiI labeled TG neurons with depolarizing current injection.

Panels A–C were recorded from a 50.5 pF TMJ neuron from a VS rat that had a resting membrane potential of -60 mV. Panels D–F are from a 50.0 pF TMJ neuron from an EC rat that had a resting membrane potential of -54 mV. The neuron from the EC rat is much more excitable than that from the VS rat. **A and D:** Action potential threshold and rheobase were determined with a 500 ms depolarizing current injection. Threshold (dotted line) is the most depolarized membrane potential achieved without firing an action potential (-24.74 mV in A; -44.67 mV in D). Rheobase is the minimum amount of depolarizing current sufficient to evoke an action potential (300 pA in A; 10 pA in D). The current trace in A contains three stimuli of 280, 290, and 300 pA. **B, C, E, and F:** The response to suprathreshold stimuli was measured as the number of action potentials evoked by 500 ms depolarizing current injections equal to 2× (B and E) and 3× rheobase (C and F).

Figure 2. N52 binding and capsaicin sensitivity

TMJ neurons are easily identified following injection of DiI into the TMJ. **A.** DiI-labeled neurons appear red under epifluorescence illumination (Arrow, Left Panel). Sections were probed with the monoclonal antibody against neurofilament 200 (N52) in order to assess the percentage of TMJ neurons that are myelinated (arrow heads, middle panel). The neuron shown is unmyelinated. **B.** Cell body size histogram of the total population of TG neurons ($n = 360$, grey bars), N52+ neurons ($n = 80$, hatched blue bars) and that of labeled TMJ neurons ($n = 103$, pink hatched bars). **C.** Typical response to a bolus application of capsaicin (500 nM) from a TMJ neuron, considered CAP+ that was harvested from a VC rat. CAP- neurons (**D**) were

easily distinguishable from CAP+ neurons in that they typically demonstrated little change in membrane potential in response to capsaicin. Scale bar in C is the same for D.

Figure 3. Examples of spontaneous activity

A. An example of low frequency (0.7 Hz) irregular spontaneous activity observed in a DiI labeled neuron (RMP = -54 mV, membrane capacitance = 44 pF) from an ES rat that was typical of the activity observed in VS, VC and ES rats. **B.** An example of higher frequency (2.5 Hz) regular activity observed in a DiI labeled neuron (RMP = -45 mV, membrane capacitance = 41 pF) from an EC rat that was typical a number of spontaneously active neurons from EC rats. Scale bar in A is the same in B.

Figure 4. Estrogen and inflammation increase the excitability of TMJ afferents

Action potential threshold, rheobase, and the response to suprathreshold stimuli were measured in TG neurons as described in Figure 1 and Methods. **A:** Estrogen and inflammation decreased action potential threshold. Values are means $\pm$ SEM. The number in parentheses reflects the number of neurons studied in each group. The number in A is the same for B and C. **B:** Estrogen and inflammation decreased rheobase, plotted here as rheobase normalized to cell capacitance (Normalized Rheobase). **C:** There was little influence of either estrogen or inflammation on the slope of the stimulus-response function. The inset in each panel reflects the results of statistical analyses of data (two-way ANOVA with Holm-Sidak post-hoc tests). *E* a significant

main effect due to estrogen, and *C* a significant main effect due to inflammation (CFA). * $p < 0.05$, ** $p < 0.01$.

Figure 5. Inflammation increases the capacitance of TMJ afferents

Capacitance was used as a measure of the cell body size of TG neurons. Two-way ANOVA revealed a statistically significant ($p < 0.01$) influence of inflammation on cell body capacitance. In order to illustrate this influence, neurons from saline (gray bars) and CFA (black bars) treated animals were pooled and plotted as a percent of the total number of neurons studied in each group (105 and 119, respectively). The median cell body capacitance of neurons from saline treated animals was 38 (with 28 and 62 as 25th and 75th percentiles), while that of neurons from CFA treated animals was 50 (with 37 and 65 as 25th and 75th percentiles).

Table 1**Spontaneous activity**

A neuron was considered spontaneously active if more than two action potentials were fired during a two-minute observation period. % SA: Percent of neurons with spontaneous activity; numbers in parentheses reflect the number of SA neurons over the number of neurons studied. Frequency: the mean instantaneous frequency of the first 10 action potentials; numbers in parentheses bracket the range of frequencies observed in each subpopulation of neurons. There was a statistically significant difference in the proportion of SA neurons among the 4 experimental groups ($p < 0.01$). There was also a statistically significant ($p < 0.01$) interaction between estrogen and inflammation with respect to their influence on resting membrane potential (RMP); where post-hoc analysis revealed a significant inflammation effect within estrogen treated animals (2) and a significant estrogen effect within saline treated animals (3). There were no statistically significant estrogen- or inflammation-induced influences on the size of SA neurons or the frequency at which SA neurons fired action potentials ($p > 0.05$, Two Way ANOVA).

Group	% SA *	Size of SA Neurons (pF)	RMP (mV) <i>I</i> **, (2,3)	Frequency (Hz)
VS	7.5 (4/53)	37.2±6.5	-52.3±3.2	0.31 (0.48-0.17)
VC	14.2 (8/56)	41.8±4.0	-45.4±2.0	0.85 (3.25-0.05)
ES	26.9 (14/52)	27.1±3.1	-44.7±1.6	0.62 (1.34-0.15)
EC	28.6 (18/63)	39.1±2.7	-49.9±1.4	2.34 (14.49-0.08)

IB4+; Neurons that were clearly stained with isolectin B4. IB4-; Neurons that were negative for IB4. Cap.+; Neurons that responded to bath application of 500 nM capsaicin with a depolarization > 20 mV. Normalized Rheobase: Rheobase normalized with respect to cell body capacitance. Data were analyzed with a two-way ANOVA; the Holm-Sidak test was used for post-hoc analysis. Values are means $\pm$ SEM. "N" number of neurons. For main or interaction effects: *E* a significant estrogen effect, *C* a significant inflammation (CFA) effect and *I* a significant interaction effect. For post-hoc analyses: "1" a significant inflammation effect within vehicle treated animals, "2" a significant inflammation effect within estrogen treated animals, "3" a significant estrogen effect within saline treated animals, and "4" a significant estrogen effect within CFA treated animals.

Table 2
Excitability Changes in Sub-Populations of TMJ Neurons Defined by IB4 and Capsaicin

Group	N	Threshold (mV)	Rheobase (pA)	Normalized Rheobase (pA/pF)
IB4+	VS	-31.3 $\pm$ 3.4	237 $\pm$ 58	3.48 $\pm$ 0.71
	VC	-35.0 $\pm$ 3.6	100 $\pm$ 62	1.63 $\pm$ 0.77
	ES	-33.2 $\pm$ 2.7	125 $\pm$ 47	2.06 $\pm$ 0.59
	EC	-37.6 $\pm$ 2.7	45 $\pm$ 57	1.04 $\pm$ 0.59
Two-way ANOVA			<i>C</i> *	<i>C</i> *
IB4-	VS	-32.8 $\pm$ 1.9	172 $\pm$ 30	2.64 $\pm$ 0.39
	VC	-41.3 $\pm$ 2.3	80 $\pm$ 27	1.09 $\pm$ 0.36
	ES	-40.9 $\pm$ 2.2	28 $\pm$ 21	0.55 $\pm$ 0.27
	EC	-39.9 $\pm$ 1.6	47 $\pm$ 20	0.69 $\pm$ 0.26
Two-way ANOVA		<i>I</i> * (<i>I</i> , 3)	<i>E</i> **, <i>I</i> * (<i>I</i> , 3)	<i>E</i> **, <i>C</i> *, <i>I</i> ** (<i>I</i> , 3)
Cap.+	VS	-35.3 $\pm$ 2.8	120 $\pm$ 31	2.44 $\pm$ 0.56
	VC	-40.8 $\pm$ 3.2	57 $\pm$ 38	0.83 $\pm$ 0.68
	ES	-35.6 $\pm$ 2.3	90 $\pm$ 27	1.50 $\pm$ 0.49
	EC	-40.7 $\pm$ 2.9	47 $\pm$ 34	0.84 $\pm$ 0.62
Two-way ANOVA		<i>C</i> *		<i>C</i> *
Cap.-	VS	-24.1 $\pm$ 3.2	410 $\pm$ 67	7.62 $\pm$ 1.21
	VC	-34.9 $\pm$ 4.5	192 $\pm$ 95	2.37 $\pm$ 1.71
	ES	-40.5 $\pm$ 3.2	80 $\pm$ 68	1.62 $\pm$ 1.21
	EC	-36.5 $\pm$ 3.5	138 $\pm$ 74	2.17 $\pm$ 1.33

Group	N	Threshold (mV)	Rheobase (pA)	Normalized Rheobase (pA/pF)
	Two-way ANOVA	E^*	E^*	E^*

* $p < 0.05$,
** $p < 0.01$.

Table 3**Passive properties**

Resting membrane potential (RMP), membrane capacitance and input resistance were assessed for each TG neuron. Data were analyzed as described for Table 2. Membrane capacitance is expressed as a median. Numbers in parentheses are 25th and 75th percentiles. Data are from non-spontaneously active neurons.

Group	N	Resting membrane potential (mV) E^*, I^{**} 1*, 2*, 3**	Membrane Capacitance (pF) C^{**}	Input resistance ($G\Omega$) C^*
VS	49	-57.5 ± 0.79	39 (28–63)	1.31 ± 0.17
VC	48	-54.8 ± 0.79	49 (33–65)	0.92 ± 0.17
ES	38	-52.9 ± 0.94	36 (29–60)	1.06 ± 0.21
EC	45	-55.6 ± 0.86	51 (42–66)	0.61 ± 0.19

Action potential waveform

Table 4

Properties of the action potential waveform were determined for each neuron as described in Methods. Data were analyzed as described in Table 2. N: the number of neurons in each group. AHP: the afterhyperpolarization magnitude. The N is the same for each parameter, except of the AHP decay rate which could not be determined for all neurons in each group. N for the AHP decay is indicated in parentheses.

Groups	N	Overshoot (mV)	Duration (ms)	Maximum rise rate (mV/ms)	Maximum fall rate (mV/ms) <i>I</i> ** ₄ **	AHP (mV)	AHP Decay (τ, ms)
VS	49	52.4 ± 1.1	5.5 ± 0.7	132 ± 11	52 ± 4	15.8 ± 0.8	114 ± 18 (44)
VC	48	54.0 ± 1.2	5.4 ± 0.7	114 ± 12	38 ± 4	17.4 ± 0.8	109 ± 19 (39)
ES	38	56.1 ± 1.4	4.4 ± 0.8	132 ± 13	45 ± 4	15.6 ± 0.9	135 ± 21 (32)
EC	45	54.0 ± 1.3	5.0 ± 0.8	141 ± 13	51 ± 4	14.8 ± 0.9	80 ± 20 (36)

Table 5
Response to suprathreshold stimuli

The response to suprathreshold stimuli was assessed by counting the number of action potentials evoked during a 500 ms depolarizing current injection equal to 2× and 3× rheobase. Neurons were then categorized as firing a single action potential or >1 action potential in response to these stimuli.

Group	N	2× rheobase stimulus % neurons firing >1 AP*	3× rheobase stimulus % neurons firing >1 AP**
VS	49	44.5	55.3
VC	48	42.5	67.5
ES	38	51.4	67.5
EC	45	71.1	89.9

* Significant difference among groups with $p < 0.05$.

** Significant difference among groups with $p < 0.01$.