Differential Effects of Estradiol on Encoding Properties of TMJ Units in Laminae I and V at the Spinomedullary Junction in Female Rats

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Central neural mechanisms are thought to play a critical role in persistent TMJD pain given that pain severity is poorly correlated with peripheral pathology (Ohrbach and Dworkin 1998) and sensitivity to stimuli involving various sensory modalities is altered (Hollins et al. 1996; Maixner et al. 1995, 1998; Sarlani and Greenspan 2005). Studies in rat and cat indicate the TMJ region is innervated mainly by small-diameter myelinated and unmyelinated fibers (Ioi et al. 2006; Kido et al. 1995; Takeuchi and Toda 2003; Takeuchi et al. 2001) that project to second-order neurons at the trigeminal subnucleus caudalis/upper cervical cord (Vc/C1–2) junction (Shigenaga et al. 1986, 1988). Lesion of the Vc/C1–2 junction, but not more rostral trigeminal brain stem areas, prevents increases in masseter muscle activity after TMJ inflammation (Hu et al. 1997). Previously, we reported in normal-cycling female rats that single TMJ units in the superficial laminae at the Vc/C1–2 junction displayed enhanced responses to intra-TMJ chemical stimulation (Okamoto et al. 2003) and increased production of c-fos positive neurons (Bereiter 2001) during proestrus, the stage of the estrous cycle associated with high circulating levels of estrogens. Although these data suggested that factors related to the estrous cycle play a significant role in modulating the activity of TMJ neurons at the Vc/C1–2 junction, it has not been determined whether changes in estrogen status per se are sufficient to account for changes in response properties observed at different stages of the reproductive cycle. Thus the aim of this study was to determine whether treatment of ovariectomized (OvX) rats with 17β-estradiol (E2), in a regimen designed to mimic both the pattern and the magnitude of circulating E2 in normal-cycling female rats, could significantly modify the properties of TMJ units at the Vc/C1–2 junction. A second aim was to compare the encoding properties of TMJ units recorded from superficial versus deep laminae at the Vc/C1–2 junction because at spinal levels the role of dorsal horn neurons in superficial versus deep laminae in nociceptive processing remains controversial (Craig 2003; Price et al. 2003).

TMJ units were activated by injection of adenosine triphosphate (ATP) directly into the joint space. Elevated levels of ATP released to the extracellular space following tissue injury have long been associated with peripheral pain (Burnstock 1996, 2000; Chizh and Illes 2001; North 2004). ATP injection into skin (Hamilton et al. 2000; Hilliges et al. 2002) or muscle (Mork et al. 2003) causes pain sensation in humans and elevated levels of ATP in synovial fluid of arthritic patients

INTRODUCTION

Temporomandibular muscle/joint disorders (TMJDS) include a family of conditions that present with pain in the temporomandibular joint (TMJ) and muscles of mastication (Dworkin and LeResche 1992; Dworkin et al. 1990). Despite substantial evidence that TMJDS is more prevalent in women than men (Bush et al. 1993; Huang et al. 2002; LeResche 1997; Lipton et al. 1993), the basis for this apparent sex difference remains uncertain. Biological factors such as estrogen status may play a significant role because the levels of clinical pain (Isselee et al. 2002; Landi et al. 2005; LeResche et al. 2003; Suenaga et al. 2001) vary over the menstrual cycle, whereas hormone replacement therapy is associated with increased risk for TMJDS after menopause (LeResche et al. 1997).
thiopental and a paralytic agent, gallamine triethiodide (15–20 mg·kg⁻¹·h⁻¹), after completion of all surgical procedures, just before the recording session. Adequate depth of anesthesia was confirmed by the absence of corneal and hindlimb withdrawal reflexes before gallamine, fully constricted pupils, and constant arterial blood pressure and heart rate throughout the experiment. Expiratory end-tidal CO₂ (3.5–4.5%) and mean arterial pressure (MAP, 100–130 mmHg) were monitored throughout the experiment. Body temperature was maintained at 38°C with a heating blanket and thermal probe.

Animals were placed in a stereotaxic frame and portions of the C1 and C2 vertebrae were removed to expose the upper cervical dorsal horn. The brain stem surface was bathed in warm mineral oil. The left temporalis muscle was reflected to expose the external pterygoid and the connective tissue overlaying the dorsal aspect of the posterior mandibular condyle. The caudal portion of trigeminal subnucleus caudalis (Vc) and the upper cervical (C1–C2) spinal cord, 4 to 7 mm caudal to the obex, was explored ipsilateral to the exposed condyle for TMJ-responsive units using the entrance of the C2 rootlet as a landmark. The rat seldom has clearly defined C1 rootlets (McLander et al. 1989); thus we refer to this region as the Vc/C1–2 junction in the text. A tangential approach (~43° off vertical, 60° off midline) was used to record single units extracellularly with tungsten microelectrodes (9 MΩ, FHC, Bowdoinham, ME). Unit activity was amplified, discriminated (model DIS-I, BAK Electronics, Mount Airy, MD), stored, and analyzed off-line on a Macintosh (Apple G4) computer using a DAQ interface board and LabVIEW software (National Instruments), as described previously (Hirata et al. 1999). Spike amplitude and shape were monitored on a digital oscilloscope and stored on tape (model CDAT4, Cygnus Technology, Delaware Water Gap, PA) for reconfirmation during off-line data analyses.

The search protocol for TMJ units began by gentle mechanical palpation of the skin and muscle overlaying the posterior aspect of the left TMJ with a cotton-tipped wooden applicator. However, the critical test to identify a TMJ unit and inclusion in further analyses was a vigorous response to direct mechanical stimulation of the exposed dorsal aspect of the posterior condyle with a blunt wooden probe (Fig. 1A; see also Fig. 1 of Okamoto et al. 2003). TMJ units were further classified by the response to convergent input from the overlying facial skin as wide dynamic range (WDR), nociceptive specific (NS), or deep only. WDR units were excited by brush (camel hair) or indentation of the skin surface with low-threshold von Frey filaments (<5 g force) and showed a greater response to press or pinch. The “press” stimulus used an arterial clip (~20 mm²) and the “pinch” stimulus used a shorter and stiffer arterial clip (~15 mm²). Brush, press, and pinch stimuli were applied for 10 s. When applied to the investigator’s skin the press stimulus was near threshold for pain sensation, although the pinch stimulus was clearly painful. TMJ units classified as NS responded vigorously to press or pinch of the skin but not to brushing. Deep-only units were activated by deep indentation of the tissues overlaying the TMJ and not to brush or pinch of the skin. The oral cavity and deep tissues remote from the head and neck were not explored routinely. No TMJ units received convergent cutaneous input activated by only brushing the skin (LTM units).

**Experimental design**

TMJ units were recorded from superficial laminae (224 ± 20 μm from penetration of the dorsal surface) and deep laminae (1,240 ± 32 μm) within 1.5 mm rostral to the level of entrance of the C2 rootlets. Because an acute angle of penetration was used, the exact vertical distance from the dorsal brain stem surface could not be determined. In most experiments only one TMJ-responsive unit was recorded in each animal preparation. However, in several cases a TMJ unit was recorded from superficial laminae and a second unit from deep laminae. The order of recording (superficial vs. deep laminae) was randomized and post hoc analysis revealed no difference in response properties compared with units from animals in which only a single unit was recorded. After confirming the response to posterior condyle
phosphate-buffered saline (PBS) or adenosine triphosphate (ATP). Injections (4–8 per unit recording session) were delivered slowly over 30 s (total volume = 20 μl) with an interinjection interval of 30 min to reduce the likelihood of tachyphylaxis. The total volume injected (160 μl) in experiments in which two units were recorded (n = 9) would have exceeded the volume of the rat TMJ space. However, a significant buildup of fluid was not likely because the rat TMJ is not a closed space and absorption kinetics for cutaneous tissues has been calculated to have a half-life of about 12 min (Roberts et al. 1997), suggesting that spread of fluid from small injection volumes (20 μl) from the joint to surrounding tissues would be minor. The protocol for chemical injections into the TMJ was: PBS (pH 7.4) followed by three successive doses of ATP (0.01, 0.1, and 1.0 mM, pH 7.4, disodium salt, Sigma). These concentrations of ATP were within the physiological range found in normal rat skeletal muscle that can rise to >5 mM after tissue injury (Morris et al. 1985). To confirm that ATP was acting through a purinergic receptor mechanism, the selective P2X receptor antagonist, pyridoxal-5-phosphate-6-azophenyl-2,4disulfonic acid (PPADS, 0.5 mM, Sigma) was coinjected with 1 mM ATP into the TMJ at the end of several (n = 20) experiments and, after 20 min, a subsequent injection of 1 mM ATP tested the recovery of evoked activity. The dose of PPADS was less than that used by Dowd et al. (1998) to block knee joint afferent nerve activity evoked by ATP after intraarticular administration. Several units in superficial laminae that were not responsive to ATP (n = 3) were tested further by injection of the small fiber excitant mustard oil (allyl isothiocyanate, 20% solution) into the TMJ at the end of the experiment and each responded vigorously.

At the end of many experiments (n = 53) TMJ units were tested for responses to jaw movement (JM). JM was produced manually by gently pulling on the lower incisor with a pair of forceps to give an incisor distance of 5–8 mm (0.5 Hz for 10 s).

Data analysis

Neural data were acquired and displayed by LabVIEW as peristimulus time histograms (PSTHs) of spikes per 1-s bins, exported to a spreadsheet and analyzed off-line. Spontaneous activity (spikes/s) was calculated as the average spike count over a 1-min epoch immediately preceding each stimulus. The evoked responses were assessed by calculating the response magnitude (Rmag), determined as the average spike count over a 1-min epoch after stimulus onset were considered unresponsive to that condition. Units were classified as ATP responsive if the total Rmag exceeded the response to PBS by >50%, independent of ATP dose. The threshold dose of ATP was defined as the lowest concentration that produced a total Rmag exceeding that to vehicle by >50%. The total Rmag to mechanical stimulation of the skin overlying the TMJ (e.g., brush, press, pinch) was determined over a 10-s stimulus period. The JM-evoked responses were determined over a 10-s stimulus period by subtracting background activity from the total spike count for each bin. The total Rmag for a given stimulus was classified as ATP responsive if the total Rmag exceeded the response to PBS by >50%, independent of ATP dose. The threshold dose of ATP was defined as the lowest concentration that produced a total Rmag exceeding that to vehicle by >50%. The total Rmag to mechanical stimulation of the skin overlying the TMJ (e.g., brush, press, pinch) was determined over a 10-s stimulus period. The JM-evoked responses were determined over a 10-s stimulus period by subtracting background activity from the total spike count for each bin. Chi-square (χ²) analysis assessed the likelihood of response to JM for the three animal groups. Total Rmag and response duration to chemical and mechanical stimuli were assessed statistically by ANOVA, corrected for repeated measures, and individual comparisons were made by Newman–Keuls after ANOVA. Fisher’s exact
probability test determined whether the frequency of occurrence of deep-only units was different from TMJ units with convergent cutaneous RFs (e.g., WDR, NS) between males and OvX females. Chi-square analysis determined whether the threshold concentration of ATP sufficient to excite TMJ units was different for males and OvX females treated with low- or high-dose E2. The cutaneous high-threshold RF areas for WDR and NS were digitized and quantified by a planimetric method using National Institutes of Health Image software (v. 1.68). Cutaneous RF areas of TMJ units from OvX females were mapped without prior knowledge of E2 treatment and compared by ANOVA. Because each female rat was given daily injections of E2, an additional group of males (n = 4) was given daily injections of sesame oil for 2 days to control for possible nonspecific effects due to handling. No differences in ATP-evoked total Rmag, response duration, or cutaneous RF areas were seen compared with males that were untreated and thus all male data were combined for the final analyses.

**Histology**

At the end of the experiment Sudan black dye (20 μl) was injected into the TMJ region through the guide cannula to verify placement in the joint space. The recording site was marked electrolytically (5 μA, 20 s). The animal was given a bolus dose of thiopental sodium (60 mg/kg, administered intravenously) and perfused through the heart with 10% formalin. Transverse sections (50 μm) were cut on a freezing microtome, stained with cresyl violet, and lesion sites were drawn onto a standard series of rat brain stem outlines (Takeshita et al. 2001).

**Immunohistochemistry**

With respect to P2X3 receptors, separate groups of HE2 and LE2 females and untreated males (n = 3 per group) were anesthetized with pentobarbital sodium (60–70 mg/kg, ip) and perfused through the heart with heparinized saline followed by 250 ml cold fixative (4% paraformaldehyde, 0.1 M phosphate, pH 7.4). The lower brain stem and upper cervical spinal cord segments were removed and postfixed in 2% paraformaldehyde, 0.1 M phosphate, pH 7.4. Sections were cut on a freezing microtome at 7 μm, slide-mounted, blocked with normal donkey serum, and incubated in anti-rabbit primary antiserum to either P2X3 (1:1,000, Chemicon, Temecula, CA) or P2X2 (1:1,000, Lincoln, NE) and incubated at 4°C overnight in primary antiserum to CA). Membranes were blocked in Odyssey Blocking buffer (Li-cor, 2001).

**TABLE 1.** Classification of ATP-responsive TMJ units in superficial and deep laminae from OvX female and intact male rats

<table>
<thead>
<tr>
<th>Laminae</th>
<th>Group</th>
<th>NS</th>
<th>WDR</th>
<th>Deep</th>
</tr>
</thead>
<tbody>
<tr>
<td>I-II (n = 55)</td>
<td>HE2</td>
<td>12 (57)</td>
<td>9 (43)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>LE2</td>
<td>11 (65)</td>
<td>6 (35)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>10 (59)</td>
<td>4 (23)</td>
<td>3 (18)</td>
</tr>
<tr>
<td>V (n = 56)</td>
<td>HE2</td>
<td>5 (20)</td>
<td>18 (72)</td>
<td>2 (8)</td>
</tr>
<tr>
<td></td>
<td>LE2</td>
<td>5 (36)</td>
<td>7 (50)</td>
<td>2 (14)</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>7 (41)</td>
<td>9 (53)</td>
<td>1 (6)</td>
</tr>
</tbody>
</table>

Number of units (% of total per group). HE2, OvX females treated with high-dose estradiol; LE2, OvX females treated with low-dose estradiol; Deep, deep RF only; NS, nociceptive-specific units; WDR, wide dynamic range units. See METHODS for further definitions of treatment groups and cell classes.

**RESULTS**

**TMJ unit classification**

In all, 111 ATP-responsive TMJ units were recorded in superficial and deep laminae at the Vc/C1–2 junction from 93 rats (Table 1). When assessed across all animal groups, TMJ units in laminae I–II were more likely to be classified as NS, whereas lamina V units were more likely to be classified as WDR (χ² = 9.36, P < 0.002). However, within individual animal groups, the relative percentages of TMJ units classified as NS or WDR in laminae I–II (χ² = 0.76, P > 0.05) and lamina V (χ² = 2.55, P > 0.05) were not different. A notable sex difference was the higher percentage (17.6%, P < 0.02, Fisher’s exact probability) of deep-only units in laminae I–II in males, whereas all TMJ units in superficial laminae from OvX rats had a convergent cutaneous RF and were classified as NS or WDR. No TMJ units were classified as LTM (i.e., displayed a convergent cutaneous RF that responded solely to innocuous brushing of facial skin). This differed from earlier studies (Kojima 1990) in which several TMJ units in Vc had an LTM-like cutaneous RF; however, in that study electrical search stimuli were used to identify TMJ units. The relative percentages of TMJ units classified as NS, WDR, or deep only in lamina V were not different between animal groups. Because the primary consideration in this study was to assess unit responses to direct TMJ stimulation, only units defined as ATP responsive (i.e., Rmag values >50% vs. vehicle injection) were included in further analyses. An additional 19 units responded to mechanical stimulation of the dorsal condyle surface, but did not respond to ATP injections. ATP-negative units were represented equally among cells classified as NS or WDR, whereas none was deep only.

**Cutaneous receptive field (RF) properties**

A total of 97 of 111 ATP-responsive TMJ units received convergent input from facial skin and were classified as NS or WDR. Figure 1A illustrates a typical NS-like response in laminae I–II in which the unit responded to press and pinch of the cutaneous RF, press of the deep tissues posterior to the mandibular condyle (D), and direct mechanical probing of the
dorsal condyle surface (C). The magnitude of the response to mechanical stimulation of the cutaneous RF of NS (Fig. 1B) or WDR (Fig. 1C) units in laminae I–II was not different across animal groups. Similarly, the magnitude of the response to mechanical stimulation of the cutaneous RF of NS or WDR units recorded in deep laminae was not different across animal groups (data not shown). In most cases, the cutaneous RF was positioned directly over the TMJ and extended anterior and ventral to the TMJ within the territories of the maxillary and mandibular branches of the trigeminal nerve (Fig. 2A). The high-threshold cutaneous RF areas of WDR units in superficial laminae from HE2 females were significantly greater than those of LE2 females (P < 0.01) or males (P < 0.01). The RF areas of NS units were similar to those for HE2 and LE2 females; however, the RF areas of NS units in HE2 females were greater than those of LE2 females.

**Estrogen status and ATP-evoked responses**

All ATP-responsive units were spontaneously active (laminae I–II: 4.3 ± 0.5 spikes/s, lamina V: 18.3 ± 2.5 spikes/s) and no significant differences in background discharge rates were seen between animal groups or different classes of TMJ units. To confirm that ATP was acting through a specific receptor mechanism, coinjection of the selective P2X receptor antagonist, PPADS (0.5 mM), with ATP into the joint space at the end of several experiments (n = 20) greatly reduced (by >70%) the ATP-evoked response in all cases followed by recovery of the evoked response after 20 min (Fig. 3). ATP-responsive neurons in superficial and deep laminae displayed dose-related increases in neural activity characterized by an increase in both firing rate and response duration (Fig. 4). The threshold dose of ATP necessary to evoke a significant increase in total Rmag was similar for HE2 and LE2 females in superficial (χ² = 2.91, P > 0.1) and deep laminae (χ² = 0.25, P > 0.1). However, compared with males, HE2 females had lower ATP thresholds for units in superficial laminae (χ² = 7.51, P < 0.006), but higher thresholds for units in deep laminae (χ² = 4.36, P < 0.037). Comparison of ATP thresholds for LE2 females and males revealed no difference for units in superficial laminae (χ² = 1.21, P > 0.1), whereas thresholds for units in deep

**FIG. 2.** Convergent cutaneous RF areas of TMJ units in superficial and deep laminae and classified as NS or WDR. A: example of cutaneous RF area overlying the TMJ region. B: average RF area for NS and WDR units in superficial laminae. C: average RF area for NS and WDR units in deeper laminae. Sample sizes: superficial laminae: HE2 NS units, n = 11; HE2 WDR units, n = 9; LE2 NS units, n = 11; LE2 WDR units, n = 5; male NS units, n = 10; male WDR units, n = 4; deep laminae: HE2 NS units, n = 4; HE2 WDR units, n = 16; LE2 NS units, n = 5; HE2 WDR units, n = 7; male NS units, n = 6; male WDR units, n = 8. **P < 0.01 vs. NS units; a = P < 0.05, b = P < 0.01 vs. HE2 females.

**FIG. 3.** Example of reduced adenosine triphosphate (ATP)–evoked response by coadministration of the selective P2X receptor antagonist PPADS (pyridoxal-5-phosphate-6-azophenyl-2,4-disulfonic acid) into the TMJ. Repeated test injections of 1 mM ATP were delivered at 30-min intervals.
laminae were lower for males ($\chi^2 = 5.24, P < 0.022$). For both HE2 and LE2 females, the threshold dose of ATP was significantly lower for units in superficial laminae than that in deep laminae (HE2, $\chi^2 = 13.64, P < 0.001$; LE2, $\chi^2 = 5.24, P < 0.022$), whereas for males the thresholds were similar for units in both regions ($\chi^2 = 1.21, P > 0.1$).

In superficial laminae, the average total Rmag for combined classes of TMJ units from HE2 females was significantly greater than that from LE2 females and males at each dose of ATP (Fig. 5, top left). Analyzing these data by cell class revealed that the increase in total Rmag for TMJ units from HE2 females was due almost completely to enhanced responses by NS units (Fig. 5, middle left), whereas the responses of WDR units were similar for all animal groups (Fig. 5, bottom left). The average total Rmag for deep-only units in lamina I–II of males was similar to that for NS and WDR units (average total Rmag to 1 mM ATP = 470 ± 189 spikes/stimulus, $n = 3$). ATP-evoked total Rmag values for TMJ units in deep laminae were similar for all animal groups when assessed for combined cell classes (Fig. 5, top right), NS units alone (Fig. 5, middle right), or WDR units alone (Fig. 5, bottom right). For HE2 females, the average total Rmag to high-dose ATP for units in superficial laminae was greater than that for units in deep laminae (817 ± 135, $n = 21$ vs. 495 ± 97, $n = 25$, $P < 0.01$). By contrast, for LE2 females, high-dose ATP evoked a greater total Rmag for units in deep than that in superficial laminae (681 ± 169, $n = 24$ vs. 314 ± 69, $n = 17$, $P < 0.01$). In males, the average total Rmag after the highest dose of ATP was lower for units in superficial than in deep laminae (303 ± 51, $n = 17$, vs. 546 ± 143, $n = 17$, spikes per stimulus, $P < 0.05$).

ATP injections produced a significant dose-dependent increase in response duration for all units. After high-dose ATP, response duration of units in laminae I–II of HE2 females (54 ± 4 s, $n = 21$) was significantly longer ($P < 0.01$) than the duration for units of LE2 females (41 ± 3 s, $n = 17$) or males (34 ± 5 s, $n = 17$). ATP-evoked increases in response duration for lamina V units were similar for all groups and averaged about 40 s. Comparisons across individual cell classes revealed no significant differences within any animal group. This suggested that the greater ATP-evoked increase in total Rmag for laminae I–II units of HE2 females (see Fig. 5) was due in part to an increase in instantaneous firing rate and to prolongation of the response. ATP injections caused a dose-dependent decrease in response latency (range = 5–8 ± 2 s after 1 mM ATP) that was similar for all classes of units in all animal groups (data not shown), suggesting similar TMJ input pathways to NS and WDR cells in both superficial and deep laminae.

Estrogen status and jaw movement–evoked responses

Jaw movement (JM) stimulation was tested on 47 TMJ units from superficial laminae in HE2 females ($n = 24$), LE2 females ($n = 11$), and males ($n = 12$). Six additional JM-responsive neurons (HE2, $n = 3$; male, $n = 3$) were recorded from deep laminae. JM-evoked activity in superficial laminae originated from articular afferents because it was blocked by lidocaine injection into the joint space (3/3 cases), whereas cutaneous input was not affected (Fig. 6A). Lidocaine reduced but did not eliminate the background discharge of TMJ units in laminae I–II (prelidocaine = 2.58 ± 0.23 vs. postlidocaine = 1.83 ± 0.19 spikes/s, $n = 12$). The percentage of JM-responsive units in superficial laminae was significantly greater for HE2 (20/24 units) or LE2 (8/11 units) females than for male rats (5/12 units) ($\chi^2 = 11.02, P < 0.004$). Both ATP-positive and ATP-negative units were excited by JM. The response pattern was consistent with the encoding of actual movement rather than jaw position because all cells adapted rapidly to sustained jaw opening. JM-evoked responses were similar across animal groups (Fig. 6B). TMJ units classified as NS or WDR displayed similar responses to JM (data not shown). However, because the search for JM-responsive units began only after testing with ATP injections was complete, we cannot exclude the possibility that prior intra-TMJ injections influenced the responses to JM.

P2X2 and P2X3 Western blots and immunohistochemistry

Western blot analysis determined the P2X2 and P2X3 receptor protein levels from separate groups of LE2 females, HE2 females, and male rats. In the trigeminal ganglion E2 treatment did not affect P2X2 [relative mean optical density (OD) LE2 = 13.47 ± 0.92; HE2 = 11.03 ± 0.53, $n = 6$]. However, P2X2 values in males (OD = 14.4 ± 0.37, $n = 6$) were marginally elevated compared with those in HE2 females ($P < 0.05$). P2X3 receptor protein levels in the trigeminal ganglion were not different between LE2 and HE2 females and males (OD = 6.69 ± 1.28, 8.49 ± 1.8, 8.71 ± 1.37, $n = 6$, respectively).

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DISCUSSION

These results indicated that estrogen status had a significant influence on the encoding properties of ATP-responsive TMJ neurons at the Vc/C1–2 junction. The nature of this influence suggested that E2 acted through multiple, yet distinct, mechanisms to modify different aspects of TMJ sensory processing. The effects of E2 were dose related and selective for specific classes of TMJ units and dependent on stimulus modality and laminar location within the dorsal horn. Such selectivity was consistent with mechanisms involving specific neural circuits and synaptic plasticity rather than generalized changes in excitability. In addition, some properties of TMJ units in laminae I–II (e.g., deep-only cells of males; jaw movement-responsive cells of OvX females) displayed sex differences, independent of E2 blood levels, consistent with a neural circuitry characterized, at least in part, by “hard-wired” differences determined during development.

Technical considerations: rationale for treatment groups

The rationale for the design of this study derived from previous results indicating that the encoding properties of TMJ units in superficial laminae from intact female rats were significantly different during the diestrous (low E2) and proestrous (high E2) stages of the estrous cycle (Okamoto et al. 1998).
approach differ from those seen in normal-cycling females or of E2 are achieved, there is evidence that results from this pellets or daily injections. Although persistent blood levels long-term (H11022 1 wk) exposure to E2 released from implanted of E2 on putative pain pathways have been examined after short-term replacement regimens. For example, long-term potentiation (LTP) in the hippocampus is greatly facilitated by 1- to 2-day exposure to E2 through N-methyl-D-aspartate (NMDA)–receptor-mediated mechanisms (Zamani et al. 2004), whereas constant exposure to E2 for 14 days had no effect on LTP (Barraclough et al. 1999). CNS responses to hypoxic stress were markedly different in OvX rats treated with short-term versus continuous E2 dosing (Buller and Day 2000). Relevant for nociceptive processing, short- versus long-term exposure to E2 had opposite effects on trkA mRNA levels in spinal dorsal root ganglion neurons (Liuzzi et al. 1999).

Estrogen status and ATP-evoked articualr input to TMJ units

Neurons in superficial and deep laminae at the Vc/C1–2 junction encoded the concentration of the ATP stimulus; however, only units in superficial laminae were enhanced by E2. Estrogen receptor (ER)–positive neurons are densely distributed in superficial laminae at the Vc/C1–2 junction (Amandusson et al. 1996; Bereiter et al. 2005), many of which display chemical phenotypes consistent with interneurons known to influence sensory input such as preproenkephalin (Amandusson et al. 1996) and γ-aminobutyric acid (GABA) (Bereiter et al. 2007). The ATP-evoked responses of TMJ units in deep laminae were not different between groups, suggesting that the relay of sensory information from superficial to deep laminae, thought to play an important role in central sensitization in cutaneous pain (Braz et al. 2005; Khasabov et al. 2002), may be less important for articular pain or not influenced by estrogen status. Furthermore, only the ATP-evoked responses of NS units in laminae I–II were enhanced by E2, whereas NS units in deep laminae and WDR units in superficial and deep laminae were similar for all groups. This differed from our previous study in which the responses to bradykinin of both NS and WDR units were enhanced during high E2 conditions (Okamoto et al. 2003). This difference may be due to a relatively higher percentage of bradykinin receptors than of P2X3 receptors on A-d fibers (Wang et al. 2006), although effects due to the hormone replacement regimen cannot be excluded. The basis for the selective enhancement of E2 on ATP-evoked activity of NS units in laminae I–II is not certain. Six of the seven P2X receptor subtypes are expressed in trigeminal ganglion cells (Ambalavanar et al. 2005; Collo et al. 1996) and nearly 50% of TMJ afferents express P2X3 receptors (Ichikawa et al. 2004; Shinoda et al. 2005) and 30% of TMJ sensory neurons are isolectin B4 (IB4) positive (Flake et al. 2004). Small-diameter sensory fibers that express IB4 project only to laminae I–II in Vc (Ambalavanar and Morris 1993; Kobayashi and Matsumura 1996) and may be the target of E2 modulation. ATP excites a high percentage of nociceptors that innervate a wide variety of tissues such as skin (Hamilton et al. 2001), muscle (Reinhoß et al. 2003), and joint (Dowd et al. 1998). Given that >80% of TMJ units were ATP responsive in this study, it is likely that ATP signaling represents a significant transduction pathway for TMJ afferents similar to that of other tissues.

Estrogen status and mechanosensory input to TMJ units

Estrogen status also modified the mechanosensory input to TMJ units and the nature of this modulation suggested mech-
anisms different from those that mediated E2 enhancement of ATP-evoked responses. First, the percentage of JM-responsive units in laminae I–II of OvX females, independent of E2 treatment, was nearly twice that of males, although the magnitude of JM-evoked activity was similar for all groups. Previously, JM-evoked c-fos production in superficial laminae was shown to depend on RGD binding integrins in OvX females, independent of the dose of E2 replacement, whereas males were not affected (Bereiter et al. 2006). ER-positive cells found in most TMJ tissues (Yamada et al. 2003) may contribute to the structural effects of estrogens (Abubaker et al. 1996; Yasuoka et al. 2000). Also, a small but significant percentage (18%) of TMJ units in superficial laminae of males received no convergent cutaneous input (“deep-only” cells), whereas no such cells were found in OvX females. Differences in the frequency of occurrence of JM-responsive and deep-only TMJ neurons between males and females, independent of E2 treatment, suggested effects due to developmental or organizational actions of sex hormones. Second, the high-threshold RF areas of WDR units in HE2 females were about 50% larger than those of LE2 females or males, whereas the magnitude of evoked responses to cutaneous pinch was similar for all groups. Overall, these data supported the notion of greater mechanosensory convergence onto TMJ units in superficial laminae of females than males.

Mechanisms of E2 modulation of ATP-evoked activation of TMJ units

The selectivity of effects of HE2 treatment on ATP-responsive neurons suggested the contribution of central neural mechanisms; however, given the widespread distribution of ER-positive cells in sensory ganglia (Bereiter et al. 2005; Sohrabji et al. 1994), dorsal horn (Amanousson et al. 1995; Bereiter et al. 2005; Papka et al. 2001), and supraspinal brain regions associated with pain modulation (Merchenthaler et al. 2004; Shughrue et al. 1997), it was not possible to identify specific sites of action after systemic administration. The case for a central mechanism was supported by two main findings: the selective enhancement of ATP-evoked responses by NS, but not WDR, units in superficial laminae, despite the fact that P2X3 receptor expression was found in superficial and deep laminae (data not shown) and, second, enlarged pinch RF areas of WDR units in superficial and deep laminae compared with LE2 females. Enlargement of high-threshold cutaneous RF areas of dorsal horn neurons after peripheral tissue injury has traditionally been explained by central mechanisms such as disinhibition (Cook et al. 1987; Hylten et al. 1989; Laird and Cervero 1989). However, in hippocampus and other forebrain areas E2 significantly altered dendritic spine density (see Cooke and Woolley 2005; McEwen et al. 2001), suggesting that structural effects of E2 also could have contributed to the changes in TMJ-evoked activity and cutaneous RF areas of Vc/C1–2 neurons. It was also possible that E2 acted at supraspinal sites outside the dorsal horn to modify chemo- and mechano-sensitive input to TMJ units. The periaductual gray (PAG) region expressed a high density of ER-positive neurons (Merchenthaler et al. 2004; Shughrue et al. 1997). In the PAG the expression of select GABA-receptor subunits varied over the estrous cycle (Griffiths and Lovic 2005), whereas short-term E2 replacement significantly modulated GABA biosynthetic enzyme activity (McCarthy et al. 1995). Although long-term E2 treatment can affect the general properties of trigeminal ganglion cells in vitro (Diogenes et al. 2006; Flake et al. 2005), it is not known whether similar changes occur normally over the estrous cycle or after short-term E2 treatment or whether these effects lead to modification of the responses to natural noxious stimuli in vivo. For example, when tested directly, glutamate-evoked TMJ (Cairns et al. 2001b) or masseter muscle (Cairns et al. 2001a) afferent nerve activity did not vary over the estrous cycle, although responses in females were greater than those in males. Only after long-term exposure to high circulating levels of E2 (>60 pg/ml) were the responses to NMDA injections by masseter muscle nerve afferents increased (Dong et al. 2007).

Interestingly, no sex- or estrous cycle–related differences were seen in the response to NMDA injection for temporalis muscle afferents (Dong et al. 2006) and in both studies no between-group differences in mechanical thresholds were noted. Earlier studies in the goat revealed only minor sex differences in the responsiveness of TMJ nociceptive afferents despite significant sex differences in the biomechanical properties of the joint (Loughner et al. 1997). In the formalin test for cutaneous pain, short-term (Kuba et al. 2006) or long-term (Mannino et al. 2007) E2 replacement in OvX rats significantly reduced formalin-induced flinching behavior in phase 2, whereas flinching during phase 1, thought to reflect peripheral sensitization, was not affected. Although P2X-positive neurons may represent only a fraction of the total number of TMJ afferents (Shinoda et al. 2005), Western blot analysis revealed no significant group differences in P2X2 or P2X3 protein levels in trigeminal ganglion samples, suggesting that changes in receptor protein could not explain the effects of E2 on ATP-evoked TMJ unit activity. Also, the threshold dose of ATP necessary to evoke TMJ unit activity in superficial or deep laminae was similar for all groups. Thus although we cannot exclude that direct action of E2 on peripheral sensory neurons contributed to the changes in TMJ unit activity at the Vc/C1–2 junction, support for such a mechanism remains uncertain.

Alternatively, E2-induced changes in the properties of peripheral or central neurons may be expressed by more indirect mechanisms such as by unmasking evoked activity. A significant percentage of articular afferents in naïve animals are characterized as “silent nociceptors” and do not respond to physiological stimuli (Michaels et al. 1996; Schable and Grubb 1993). One possible consequence of changes in estrogen status may be to unmask these silent afferents (Michaelis et al. 1996) or silent synapses within the CNS (Kerchner et al. 1999). An E2-induced increase in the number of joint- or muscle-responsive afferents could account for increases in EMG activity without necessarily lowering the threshold or increasing the maximum response of single afferents to a given stimulus (Cairns et al. 2001a). Unmasking silent afferents also could account for the increased number of Fos-positive neurons at the Vc/C1–2 junction after TMJ injury during high E2 conditions (Bereiter 2001). The sensitization of dorsal horn neurons after tissue injury (Ji et al. 2003; Woolf and Salter 2000) and alterations in central neural excitability after changes in estrogen status (Malyala et al. 2005; McEwen 2001) may share common mechanisms (e.g., increased NMDA receptor and MAP kinase activity, disinhibition of GABAergic activity);
however, the details of this apparent overlap are not yet defined. Because the present study used rats after acute surgical exposure of the TMJ region, it cannot be excluded that some properties of these units were altered. Indeed, application of lidocaine to the joint space inhibited JM-evoked activity and condyle-evoked activity (not shown) and reduced the background firing rate (Fig. 6A).

Role of superficial and deep laminae in TMJ nociceptive processing

The role of different classes of neurons in superficial and deep laminae in nociceptive processing remains controversial (Craig 2003; Price et al. 2003); however, this controversy has been based mainly on models of cutaneous rather than articular pain. Craig (2003) proposed that nociceptive lamina I units are modality specific, receive only monosynaptic input from A-δ and C-fibers, and serve an interoceptive function, whereas lamina V neurons receive convergent input from A-β, A-δ, and C-fibers and serve sensorimotor integrative functions. By contrast, others have reported (Braz et al. 2005; Eckert et al. 2006; see Willis et al. 2002) that nociceptive neurons in both lamina I and lamina V encode multiple stimulus modalities across a range of intensities consistent with a role in mediating sensory aspects of nociceptive processing. Our results generally support this latter view. TMJ units in lamina I and V encoded the intensity of both mechanical and chemical stimulus modalities, properties consistent with a role in mediating spontaneous and movement-evoked jaw pain. Earlier recording studies of TMJ units used female (Kojima 1990) or male rats (Nishikawa et al. 2004) or cats of either sex (Broton et al. 1988) and combined the results from superficial and deep laminae. Similarly, most studies of joint-responsive units at spinal levels used only male animals and grouped the data from superficial and deep laminae (see Schaible 2004; Schaible and Grubb 1993), suggesting that selective effects of estrogen status on second-order neurons in superficial laminae of the dorsal horn would not have been detected. TMJ units were not tested for ascending projections, as in our previous study in which about 15% of lamina I neurons were driven antidromically from posterior thalamus (Takeshita et al. 2001), leaving open the possibility that estrogen status may have different effects on projection and nonprojection neurons. Based on encoding properties and the influence of estrogen status, we propose that TMJ units in laminae I and V share some functional roles in nociception such as sensory discrimination; however, lamina I units serve additional roles. Consistent with the view of Craig (2003), TMJ neurons in superficial laminae may play a significant role in monitoring homeostatic conditions, in this case estrogen status. Lamina I cells also may form part of an ascending pathway that recruits supraspinal pain controls that, in turn, modulates sensory input to deep dorsal horn neurons (McMahon and Wall 1988; Suzuki et al. 2002). Sex differences in the recruitment of supraspinal control systems have been proposed as constituting a contributing factor in TMJD pain (Bradgon et al. 2002). Purinergic mechanisms may be particularly involved in endogenous pain controls because P2X receptors in caudal Vc were necessary for the initiation and maintenance of central sensitization of neurons in rostral portions of the trigeminal brain stem complex after tooth pulp injury (Chiang et al. 2005; Hu et al. 2002).

In conclusion, convergence of sensory signals from articular chemo- and mechanoreceptors and cutaneous mechanoreceptors onto single neurons at the Vc/C1–2 junction in an estrogen-dependent manner supports the hypothesis that lamina I neurons play a critical role, distinct from lamina V neurons, in mediating different aspects of spontaneous and evoked pain in TMJD.

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