Response Properties of TMJ Units in Superficial Laminae at the Spinomedullary Junction of Female Rats Vary Over the Estrous Cycle

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Okamoto, K., H. Hirata, S. Takeshita, and D. A. Bereiter. Response properties of TMJ units in superficial laminae at the spinomedullary junction of female rats vary over the estrous cycle. J Neurophysiol 89: 1467–1477, 2003; 10.1152/jn.00795.2002. Neurons responsive to stimulation of the temporomandibular joint (TMJ) region were recorded from superficial laminae at the trigeminal subnucleus caudalis/upper cervical cord (Vc/C2) junction region of cycling female rats under barbiturate anesthesia. To determine if receptive field (RF) properties or sensitivity to algesic chemicals of TMJ units vary over the estrous cycle, animals were selected from proestrous (high estrogen) or early diestrous (low estrogen) stages. More than 90% of TMJ units from each group received convergent nociceptive input [wide dynamic range (WDR) or nociceptive specific (NS)-like] from facial skin. The cutaneous high-threshold RF areas of WDR units from proestrous rats were 30% larger than diestrous units, while RF areas of NS units were similar. Bradykinin (BK, 0.1–10 μM) injection into the TMJ region excited a high percentage of units (>80% of total) from both groups in a dose-related manner. However, BK-evoked response magnitude (Rmag, +140%) and duration (+64%) were greater for proestrous than diestrous units. Both WDR and NS-like TMJ units of proestrous females displayed enhanced BK-evoked Rmag values and response duration. Glutamate or mustard oil excitation of TMJ units was not affected by stage of the estrous cycle. Several TMJ units from proestrous and diestrous females were activated antidromically from the contralateral posterior thalamus, indicating that projection and nonprojection units were included in the sample population. These results were consistent with the hypothesis that factors related to stage of the estrous cycle modify the processing of deep craniofacial inputs by superficial dorsal horn neurons at the spinomedullary junction, a key region for the initial integration of sensory signals from the TMJ.

INTRODUCTION

Temporomandibular disorders (TMD) comprise a family of conditions that often present with diffuse pain in the temporomandibular joint (TMJ) region and masticatory muscles that spreads to adjacent areas of the face and neck (Denucci et al. 1996; Dworkin and LeResche 1992; Turp et al. 1998). Although the basis for TMD pain is not well defined, the signs and symptoms suggest a prominent role for central neural mechanisms. TMD patients display lowered sensory thresholds, altered temporal summation to experimentally induced pain (Maixner et al. 1995, 1998), and a reduced capacity to recruit endogenous pain inhibitory controls (Bragdon et al. 2002).

Women are over-represented for TMD (Bush et al. 1993; LeResche 1997; Stohler 1997) and other painful arthritic/arthralgic conditions (Berkeley 1997; Buckwalter and Lappin 2000; Fillingim and Maixner 1995; Gabriel 2001; Unruh 1996). A role for sex hormones in TMD pain is supported by reports of higher prevalence among women of reproductive age that diminishes after menopause and recurs after estrogen replacement therapy (LeResche et al. 1997). Also, pain due to TMJ disk displacement (Suenaga et al. 2001) and muscle pressure pain thresholds vary systematically over the menstrual cycle (Isselée et al. 2002). Numerous animal studies have demonstrated that pain-like behavior evoked by cutaneous (Martinez-Gomez et al. 1994), deep, or visceral tissue stimulation (Giamberardino et al. 1997; Kayser et al. 1996; Ness et al. 2001) varies over the estrous cycle. However, few experimental studies have examined the effect of reproductive status on nociceptive processing of input from articular tissues. Current understanding of the central neural basis for articular pain derives mainly from studies using the knee and ankle joints as models (see Kidd et al. 1996; Schaible and Grubb 1993), while fewer studies have used models specifically related to the TMJ region (see Sessle et al. 1993). The TMJ capsule, masticatory muscles, and overlying facial skin are supplied by branches of the trigeminal, upper cervical, and vagus nerves (Casati et al. 1999; Denny-Brown and Yanzawa 1973; Kido et al. 1993; Klineberg 1971; Uddman et al. 1998; Widenfalk and Wiberg 1990). Converging lines of evidence indicate that the subnucleus caudalis/upper cervical cord junction region (Vc/C2 region) plays a significant role in nociceptive processing related to the TMJ. Afferent nerves that supply the TMJ region terminate in common laminae at the Vc/C2 junction region (Jacquin et al. 1983; McNeill et al. 1991; Pfälzer and Arvidsson 1988). Selective lesion of the Vc/C2 junction region, but not of more rostral areas of the trigeminal sensory complex, blocks the increase in masseter muscle activity caused by inflammation of the TMJ region (Hu et al. 2002).

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methohexital and a paralytic agent, gallamine triethiodide (15–20 mg/kg) was maintained at 38°C.

We reported that TMJ units in superficial laminae at the Vc/C2 junction region of male rats were excited by bradykinin (BK) injection into the joint space in a dose-dependent manner (Takeshita et al. 2001). BK is a potent allogenic agent whose levels are elevated after acute or chronic inflammation (Hargreaves et al. 1988) as well as within the TMJ after acute injury (Swift et al. 1998). The present study used localized injections of BK in the TMJ region of cycling female rats to test the hypothesis that the encoding properties of TMJ units in superficial laminae at the Vc/C2 junction region vary over the estrous cycle.

**Methods**

The protocols were approved by the Institutional Animal Care and Use Committee of Rhode Island Hospital and conformed to the established guidelines set by The National Institutes of Health for the care and use of laboratory animals (PHS Law 99-158, revised 2002). Beginning at 7–10 days prior to the experiment, the stage of the estrous cycle was determined by daily microscopic examination of vaginal smears taken by gentle lavage. Females were selected in proestrus or early diestrous stages of the estrous cycle when sex steroid levels are expected to be at the highest and lowest, respectively (Smith et al. 1975). The stage of estrous cycle was determined by monitoring vaginal smear cytology daily for two consecutive cycles and defined as proestrus or early diestrous (metestrous) only if normal cycling was confirmed and on the day of the experiment >70% of total cells were the expected cell type (see Adler 1981).

**Animal preparation**

Female rats (Sprague-Dawley, Harlan, 250–300 g) were anesthetized initially with pentobarbital sodium (60 mg/kg, ip). After tracheostomy, animals were resired artificially with oxygen-enriched room air and catheters were placed in the right femoral artery (blood pressure monitor) and right jugular vein (anesthesia and drug infusions). Anesthesia was maintained by a continuous infusion of methohexital sodium (18–25 mg kg⁻¹ h⁻¹) and switched to a mixture of methohexital and a paralytic agent, gallamine triethiodide (15–20 mg kg⁻¹ h⁻¹) after completion of all surgical procedures, just prior to the recording session. Adequate depth of anesthesia was confirmed by absence of corneal and hindlimb withdrawal reflexes prior to galvanic, fully constricted pupils, and constant arterial blood pressure and heart rate throughout the experiment. The animal was placed in a stereotaxic frame and the C1 and a portion of the C2 vertebrae were removed to expose the upper cervical dorsal horn. The brain stem surface was bathed in warm mineral oil. A portion of parietal bone was removed from the right skull, contralateral to the recording electrode, for placement of a dual stimulating electrode array in the sensory thalamus. The left temporalis muscle was reflected partially to expose the external pterygoid muscle and connective tissue overlying the dorsal surface of the posterior mandibular condyle. 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.

**Electrophysiology**

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 for TMJ-responsive units using the entrance of the C2 rootlet as a landmark. A tangential approach (approximately 45° off vertical, 60° off midline) was used to record single units extracellularly with tungsten microelectrodes (9 MΩ, Frederick Haer, Bowdoinham, ME) within 500 μm after surface penetration. Unit activity was amplified, discriminated (model DIS-1, BAK Electronics, Mount Airy, MD), and then stored and analyzed off-line on an Macintosh (Apple G3) computer using a DAQ interface board and LabVIEW software (National Instruments), as described previously (Hirata et al. 1999). The window discriminator allowed single units to be isolated on the basis of amplitude and time. Spike amplitude and shape were monitored throughout each experiment and displayed on a digital oscilloscope and stored on tape (model CDAT4, Cygnus Technology, Delaware Water Gap, PA) for subsequent reconfirmation during off-line data analyses. To confirm that only a single spike contributed to the overall spike count during each stimulus trial, a discriminated action potential waveform was displayed continuously on a digital oscilloscope and compared against the original shape and amplitude for that spike at the outset of the recording period.

The search for single units began by gentle mechanical palpation of the skin over the posterior aspect of the TMJ with a cotton-tipped applicator and then with a small metal burnisher (site D in Fig. 1A). However, the critical test for inclusion of a unit for further analysis was a vigorous response to direct probing of the surgically exposed dorsal aspect of the posterior condyle and surrounding muscles with a blunt metal probe (site C in Fig. 1). TMJ units that received convergent input from facial skin were classified further as wide dynamic range (WDR) or nociceptive specific (NS). WDR units were excited by brush (camel hair) or indentation of the skin surface with low-threshold von Frey filaments (1.2 g force) and showed a greater response to press or pinch (see example in Fig. 1B). An arterial clip (approximately 20 mm²) was used for “press” stimuli, while “pinch” stimuli were delivered by a shorter, more stiff arterial clip (approximately 15 mm²). Brush, press, and pinch stimuli were applied for 10 s. When applied to the investigator’s skin, the intensity of the press stimulus was near threshold for pain sensation, while the pinch stimulus was clearly above threshold. NS units were activated by press or pinch of the skin but not by brushing. TMJ units activated by deep indentation of the TMJ region alone with no apparent cutaneous receptive field (RF) were classified as deep-only cells. However, no systematic attempt was made to stimulate deep tissues distant from the TMJ region or within the oral cavity. No TMJ units were found that also responded only to brushing the skin [i.e., low threshold mechanoceptive (LTM) units]. Electrical stimuli (0.1–5 mA, 0.2–2.0 ms, square-wave pulses) were applied to the TMJ region by a dorsal approach to estimate the type of fiber input. A bipolar electrode (Frederick Haer; 2 mm separation) was inserted approximately 2 mm deep into the tissue surrounding the posterior aspect of the mandibular condyle after retracting the temporalis muscle.

**Experimental protocol**

A single TMJ-responsive unit was recorded from each animal preparation. After confirming the response to condyle stimulation, the surface of the neck on the face was marked. Stormont (33 gauge) was injected from the end of the guide cannula. Test solutions were made from the end of the guide cannula. Test solutions were made from phos- phate-buffered saline (PBS), glutamate (GLU), BK, and mustard oil (MO). Each solution was injected from a separate inner cannu-
repeated injections of 1\(\mu\)l each unit. In pilot studies (Typically, four to eight injections were made while recording from interval of 30 min to minimize tachyphylaxis, especially for BK. Slowly over 30 s (total volume tubing assembly to prevent drug mixing. Injections were delivered posterior mandibular condyle; SA, spontaneous activity. C, probe of muscle and connective fascia directly overlying the exposed press of skin; PI, pinch of skin; D, deep probe through skin into TMJ region; neous activity. Abbreviations: B, brush of skin overlying TMJ region; PR, Stimuli were applied manually for 10 s. * Responded to drugs. ** Increased activity by a second investigator without knowledge of the stage of cycle and found not to differ significantly. The frequency distributions of RF areas for proestrous and diestrous females. Cutaneous high-threshold RF areas were determined using a small serrated forceps (approximately 3 mm\(^2\)) and mapped onto a planimetric method using National Institutes of Health Image software (v. 1.61). Cutaneous high-threshold RF areas were not mapped blindly; how- ever, in several cases the RF area was confirmed by a second investigator without knowledge of the stage of cycle and found not to differ significantly. The frequency distributions of RF areas for proestrous and diestrous units were compared using the K-S two-sample test.

**Antidromic stimulation**

An array of two concentric bipolar stimulating electrodes (SNE- 100, Rhodes Medical Instruments, Tujunga, CA), separatedrostro-caudally by 2 mm, was directed at the contralateral ventral postero-medial nucleus (VPM), and posterior nucleus group (PO) of the thalamus. The approximate stereotaxic coordinates in millimeters for VPM were AP = -2.5 from bregma, ML 2.5, DV -4 to 9 from the cortical surface; for PO, AP = -4.5, ML 2.5, DV 4–9. Antidromic activation was defined by a constant latency (<0.1 ms jitter), high-frequency following (0.1-ms pulse, 200–300 Hz, 20-ms train duration), and collision with an orthodromically driven spike within a critical time interval. The maximum allowable current used to define an antidromic response was 500 \(\mu\)A. Brain loci for antidromic responses were marked electrolytically (30 \(\mu\)A, 30 s).

**Data analysis**

Neural activity and arterial pressure 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 by averaging the spike counts during a 1-min epoch immediately preceding each stimulus. Evoked responses were assessed statistically by first calculating the response magnitude \((R_{max})\). \(R_{max}\) was determined by subtracting the mean plus two times the standard deviation (SD) of the background activity from the total spike count for each bin. The total \(R_{max}\) for a given stimulus was defined as the cumulative sum of spikes for those contiguous bins in which the spike count minus the background gave a positive value. The total \(R_{max}\) can be viewed as equivalent to the “area under the curve” for each stimulus (compare, for example, Fig. 4, A and B). Accordingly, response duration was defined as the time interval after stimulus onset until three consecutive bins with a positive spike count occurred above background (initial latency) and until the value of three consecutive bins no longer exceeded the mean + 2SD above background activity. This approach was described previously for caudal VC units responsive to corneal stimuli (Hirata et al. 1999). A unit that failed to show three consecutive bins with a positive \(R_{max}\) value within 100 s after the onset of the stimulus was considered unresponsive to that condition. Units were classified as BK-responsive (BK\(^+\) units) if the total \(R_{max}\) exceeded that after injection of PBS by >50%, independent of BK dose. The threshold concentration of BK was defined as the lowest concentration that produced a total \(R_{max}\) that exceeded that to PBS by >50%. Responses to mechanical and chemical stimuli were assessed statistically by analysis of variance (ANOVA) corrected for repeated measures (Winer 1971) and individual comparisons were made by Newman-Keuls after ANOVA. Chi-square analysis determined if different classes of neurons (e.g., WDR, NS, deep only) or frequency of responsiveness to different test chemicals were represented equally between proestrous and diestrous females. Cutaneous high-threshold RF areas were determined using a small serrated forceps (approximately 3 mm\(^2\)) and mapped onto a series of standardized rat face outlines (see examples in Figs. 4, 5, and 6). No attempt was made to selectively map the low-threshold RF area for WDR units. The RF areas were digitized and quantified by a planimetric method using National Institutes of Health Image software (v. 1.61). Cutaneous RF areas were not mapped blindly; how-
while the mean RF area for NS and WDR units were compared by ANOVA.

Histology

At the end of the experiment Sudan black dye (20 μl) was injected into TMJ region through the guide cannula to verify placement in the joint space. The animal was given a bolus dose of methohexital sodium (60 mg/kg, iv) and perfused through the heart with 10% formalin containing potassium ferrocyanide to identify antidromic stimulation sites by the Prussian blue reaction. Recovered sites were drawn on a standard series of brain outlines adapted from the atlas of Paxinos and Watson (1997). The recording site was marked electrolytically (<5 μA, 20 s, see Fig. 7A in Takeshita et al. 2001).

RESULTS

General properties

A single neuron from each of 46 proestrous and 33 diestrous female rats was recorded from the superficial laminae at the Vc/C2 junction region as defined by a vigorous response to probing the dorsal aspect of mandibular condyle (Fig. 1). All TMJ units from proestrous (4.5 ± 0.5 spikes/s) and diestrous (4.6 ± 0.6 spikes/s) rats were spontaneously active and most displayed an irregular burst-like pattern of activity. Spontaneous activity rates for different classes of units (e.g., WDR, NS, deep only) were similar. Since mechanical stimuli were used to classify TMJ units, it was necessary to determine if stage of the estrous cycle affected mechanical sensitivity. As seen Fig. 1C, TMJ units classified as WDR-like from proestrous and diestrous rats encoded mechanical stimuli with a progressive increase in firing rate with increasing stimulus intensity. Proestrous units displayed a greater numerical increase in firing than diestrous units; however, this difference was not significant. No TMJ units were inhibited by mechanical stimulation of deep tissues in TMJ region or the overlying facial skin. The average depth of recording for proestrous (192 ± 12 μm) and diestrous units (208 ± 30 μm) was similar and all sites were at or within 1.5 mm rostral to the entrance of the C2 rootlets (Fig. 2). Nearly all TMJ units recorded from proestrous (42 of 46, 91.4%) and diestrous females (30 of 33, 90.8%) also received convergent nociceptive input from the overlying facial skin, while the remaining cells were classified as deep only (see Table 1). Although a greater percentage of WDR units was sampled from proestrous than diestrous females, the relative percentage of WDR, NS, and deep cells was not significantly different (χ² = 4.37, df = 2, P > 0.05). The depth reading from the microdrive indicated that, on average, WDR units were recorded from deeper locations in the dorsal horn than NS units in both animal groups (Fig. 2B).

TMJ units from proestrous females had significantly larger cutaneous high-threshold RF areas than units from diestrous animals (Fig. 3A, χ² = 8.23, df = 2, P < 0.02). The average cutaneous RF area of proestrous units was 30.1% greater than diestrous units. This difference was due mainly to enlarged RF areas for WDR units, while the RF areas for NS units were similar for proestrous and diestrous females (Fig. 3B). Typically, the convergent cutaneous RF extended rostral and ventral from the TMJ region to include the maxillary and mandibular divisions of the trigeminal nerve (see examples in Figs. 4C and 5C). In all but two cases, at least a portion of the cutaneous RF was above the deep RF for the TMJ region. Although not tested extensively in all cases, no units were activated by mechanical stimulation of the contralateral TMJ region or facial skin.

A bipolar stimulating electrode was inserted into the TMJ region to estimate the type of fiber input that relayed information from the TMJ to the Vc/C2 region. In both proestrous and 

TABLE 1. Cell classification, frequency of occurrence of chemical responses, and general properties of TMJ units recorded in laminae I–II at the Vc/C2 junction from proestrous and diestrous female rats

<table>
<thead>
<tr>
<th>Cell classification</th>
<th>Proestrus</th>
<th>Diestrus</th>
</tr>
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<tbody>
<tr>
<td>WDR</td>
<td>23/46 (50)</td>
<td>9/33 (27.3)</td>
</tr>
<tr>
<td>NS</td>
<td>19/46 (41.3)</td>
<td>21/33 (63.6)</td>
</tr>
<tr>
<td>Deep only</td>
<td>4/46 (8.7)</td>
<td>3/33 (9.1)</td>
</tr>
<tr>
<td>PBS</td>
<td>36/46 (78.3)</td>
<td>19/32 (59.4)</td>
</tr>
<tr>
<td>BK*</td>
<td>38/46 (82.6)</td>
<td>25/30 (83.3)</td>
</tr>
<tr>
<td>Glu</td>
<td>9/22 (40.9)</td>
<td>11/17 (64.7)</td>
</tr>
<tr>
<td>MO</td>
<td>29/31 (93.5)</td>
<td>17/22 (77.3)</td>
</tr>
<tr>
<td>Spontaneous activity (spikes/s)</td>
<td>4.5 ± 0.5</td>
<td>4.6 ± 0.6</td>
</tr>
<tr>
<td>Recording depth (μm)</td>
<td>192 ± 21</td>
<td>208 ± 30</td>
</tr>
</tbody>
</table>

Values are number of rats with percentages in parentheses, except for Spontaneous activity and Recording depth, which are mean ± SE. TMJ, temporomandibular joint; WDR, wide dynamic range; NS, nociceptive specific; PBS, phosphate-buffered saline; Glu*, glutamate; MO, mustard oil.

Fig. 2. Recording locations of TMJ units classified according to cutaneous RF properties. A: map of recording location for proestrous (left) and diestrous (right) units. Note that all recording sites recovered by histological inspection were located within the superficial laminae. P5 and P6 refer to approximate distance in millimeters from the obex. B: average recording depth for WDR and nociceptive specific (NS)-like units from proestrous and diestrous animals. *P < 0.05, **P < 0.01 vs. WDR units.
Diestrous females input was mainly from small myelinated A-delta fibers since the conduction velocities averaged 3.9 ± 0.3 and 4.5 ± 0.5 m/s, respectively. Only 6 of 74 TMJ units (proestrus, n = 2; diestrus, n = 4) displayed long-latency activity (<2 m/s conduction velocity) consistent with C-fiber input, assuming a distance of 20 mm from the dorsal condyle surface to the Vc/C2 junction region.

Responses to PBS and BK

Injection of PBS alone activated 36 of 46 (78.3%) TMJ units in proestrus and 19 of 32 (59.4%) units in diestrous females (i.e., $R_{mag} > 10$ spikes per stimulus). Thirty-eight of 46 (82.6%) units from proestrous and 25 of 30 units (83.3%) from diestrous females were excited by BK injection into the TMJ region. $BK^{+}$ units (i.e., total $R_{mag} > 50\%$ above that to PBS injection) were represented among all cell classes of proestrus (WDR, n = 19; NS, n = 16; deep only, n = 3) and diestrous females (WDR, n = 6; NS, n = 18; deep only, n = 1). The lowest concentration of BK (0.1 $\mu$M) tested was sufficient to excite 59.1% of BK$^{+}$ units in proestrus females and 87.5% of BK$^{+}$ units in diestrous females. Figures 4 and 5 present examples of BK$^{+}$ units from a proestrous and diestrous female, respectively. The raw spike counts are shown in Figs. 4A and 5A with confirmation of constant spike shape and amplitude for each unit. The $R_{mag}$ values (spike count minus background activity) are displayed in Figs. 4B and 5B. Co-injection of the selective $B_2$ receptor antagonist, HOE 140, greatly reduced BK-evoked activity supporting the notion of a receptor-mediated response (Fig. 6A). Each of five units tested from proestrous females displayed a decreased response of >95%...
after co-injection of BK plus HOE 140 compared with BK alone (not shown).

Figure 7 compares the average total $R_{mag}$ for all BK+ units (top, i.e., WDR, NS, and deep only cells), WDR units alone (middle), and NS units alone (bottom) from proestrous and diestrous females after injection of PBS and cumulative doses of BK. The total $R_{mag}$ for proestrous units increased to near-maximal values after only 0.1 $\mu$M BK and remained elevated after higher doses and was similar for WDR and NS units. The total $R_{mag}$ among all proestrous units was significantly greater than that of diestrous units at the lowest (0.1 $\mu$M) and highest doses of BK (10 $\mu$M). The largest overall difference in total $R_{mag}$ between proestrous and diestrous units occurred among WDR cells (Fig. 7, middle). At each cumulative dose of BK the total $R_{mag}$ for WDR units from proestrous units was significantly greater than for diestrous units ($P < 0.05$). By contrast, the increase in total $R_{mag}$ among NS units was not different for proestrous and diestrous units over the cumulative doses of BK (Fig. 7, bottom). Figure 8 compares the response duration for all BK+ units (top, i.e., WDR, NS, and deep only cells), WDR units alone (middle), and NS units alone (bottom) from proestrous and diestrous females after injection of PBS and cumulative doses of BK. The response duration for all proestrous units increased progressively with increasing doses of BK, while the maximum increase in response duration for diestrous units was seen after 1 $\mu$M BK. Response duration evoked by the highest dose of BK (10 $\mu$M) was significantly longer for proestrous than diestrous units and this effect occurred among WDR and NS units.

Responses to GLU and MO after BK

Injection of GLU (200 mM) into the TMJ region, after cumulative doses of BK, excited many proestrous (40.9%) and diestrous units (64.7%). Examples of responses to GLU after BK for proestrous and diestrous units are shown in Figs. 4 and 5. To determine the relative responses of TMJ units to BK, after co-injection of BK plus HOE 140 compared with BK alone (not shown).

Figure 7 shows the average total $R_{mag}$ for all BK+ units (top, i.e., WDR, NS, and deep only cells), WDR units alone (middle), and NS units alone (bottom) from proestrous and diestrous females after injection of PBS and cumulative doses of BK. The total $R_{mag}$ for proestrous units increased to near-maximal values after only 0.1 $\mu$M BK and remained elevated after higher doses and was similar for WDR and NS units. The total $R_{mag}$ among all proestrous units was significantly greater than that of diestrous units at the lowest (0.1 $\mu$M) and highest

**Figure 6. Effect of BK alone and BK plus $B_2$ receptor antagonist injection into the TMJ region.** A: oscillographic records of responses to PBS, BK, and co-injection of BK plus the selective $B_2$ antagonist, HOE-140 (10 $\mu$g/20 $\mu$L). Note also that 20% mustard oil (MO) caused a marked increase in activity after HOE-140, suggesting that $B_2$ receptors are not necessary for the response to MO. B: WDR-like cutaneous (shaded) and deep (black) RF areas. C: recording site. Other abbreviations as in Fig. 4.
GLU, and MO, the average total $R_{mag}$ caused by injection of each agent was compared among BK/H11001 units (Fig. 9). Within each animal group the peak total $R_{mag}$ to BK, independent of BK dose, was not different from for GLU, while the total $R_{mag}$ after MO was significantly ($P < 0.01$) greater than after BK or GLU. Between group comparisons (i.e., proestrous versus diestrous) of the total $R_{mag}$ after BK was greater for proestrous

![Diagram of BK effect on response duration of TMJ units](http://jn.physiology.org/)

**Fig. 7.** Effect of BK on total $R_{mag}$ for TMJ units defined as BK$^+$ from proestrous and diestrous females. Top: $R_{mag}$ for all BK$^+$ units (i.e., WDR, NS, and deep units); middle: $R_{mag}$ for WDR units alone; bottom: $R_{mag}$ for NS units alone. Total $R_{mag}$ is defined as (total spikes/stimulus - mean + 2SD of background). Sample size: total proestrous units, $n = 38$; total diestrous units, $n = 20$. *$P < 0.05$, **$P < 0.01$ vs. response to PBS (0 uM BK); a = $P < 0.05$, b = $P < 0.01$ vs. diestrous.

![Diagram of BK effect on response duration of TMJ units](http://jn.physiology.org/)

**Fig. 8.** Effect of BK on response duration of TMJ units defined as BK$^+$ from proestrous and diestrous females. Top: duration for all BK$^+$ units (i.e., WDR, NS, and deep units); middle: duration for WDR units alone; bottom: duration for NS units alone. Response duration is defined as the time after stimulus onset in which at least 3 contiguous bins contained positive values in excess of the mean + 2SD of the background activity. Sample size: total proestrous units, $n = 38$; total diestrous units, $n = 20$. *$P < 0.05$, **$P < 0.01$ vs. response to PBS (0 uM BK); a = $P < 0.05$, b = $P < 0.01$ vs. diestrous.
The average current for activation was 178 μA for proestrous and diestrous units, respectively.

FIG. 9. Comparison of total \( R_{mag} \) (spikes/stimulus — background) for TMJ units (data not shown). A majority of TMJ units from proestrous (93.5%) and diestrous females (77.3% of units tested) responded vigorously to MO as shown by the example in Fig. 6A. This example also suggested that MO-evoked TMJ unit activity was not dependent on the bradykinin B₂ receptor since responses occurred within 60 min after administration of HOE 140. The total \( R_{mag} \) to MO among proestrous and diestrous units was not different among TMJ units classified as BK⁺. Correlation analyses revealed that the total \( R_{mag} \) to BK was not correlated with the response to GLU or MO (Spearman rank-order analysis, \( r_s < 0.3 \)) for either proestrous or diestrous units (data not shown).

Antidromic responses

Several TMJ units from each animal group were tested for antidromic activation from the contralateral sensory thalamus. Five of 20 (25%) units from proestrous females and 3 of 7 (43%) from diestrous females were activated antidromically from the posterior thalamus. Successful activation of units occurred only from the caudal-most electrode of the array, centered in the PO, and none from the more rostral electrode that passed through the ventro-posteromedial nucleus were consistent. Effective antidromic sites in PO were similar to those that activated cornea-responsive units recorded from caudal Vc (see example in Fig. 7 of Hirata et al. 2000). The average latency for activation from the PO was similar for proestrous (5.9 ± 0.3 ms) and diestrous units (7.3 ± 1.0 ms) units. The average current for activation was 178 ± 64 and 250 ± 95 μA for proestrous and diestrous units, respectively.

DISCUSSION

Three main findings supported the hypothesis that the response properties of TMJ units in superficial laminae at the Vc/C₂ junction region vary over the estrous cycle. First, injection of low doses of BK (0.1 μM) into the TMJ region evoked greater increases in total \( R_{mag} \) (i.e., total spikes per stimulus above background) and response duration of proestrous than diestrous units. Second, injection of high doses of BK (10 μM) caused further increases in total \( R_{mag} \) and response duration above that seen after lower doses in proestrous units, while diestrous units displayed reduced responses consistent with desensitization. Third, convergent cutaneous high-threshold RF areas for proestrous units were larger than diestrous units, due mainly to an increase in RF area for WDR-like units. These results indicated that TMJ unit responses to chemical and mechanical stimulus modalities, especially among WDR-like units, were enhanced during proestrus, a stage of the estrous cycle when circulating levels of sex steroids normally are elevated.

These results extend those of a previous study in which the RF properties of TMJ units in superficial laminae at the Vc/C₂ junction region of male rats were determined (Takeshita et al. 2001). TMJ units from proestrous and diestrous females shared many properties with those of males; however, there were differences. More than 90% of TMJ units from proestrous or diestrous females received convergent nociceptive (WDR or NS-like) input from facial skin, while only 74% of units from males were classified as WDR or NS (\( \chi^2 = 14.8, df = 4, P < 0.01 \)). This suggested that the degree of spatial convergence onto second-order TMJ units was greater for females than males and did not depend on stage of the estrous cycle. Post-hoc analyses of cutaneous RF areas for WDR units from males (1.4 ± 0.18 cm², \( n = 19, P < 0.01 \)) were smaller than those from proestrous but not diestrous units. The percentages of TMJ units responsive to BK, GLU, or MO were similar in females and males. However, post-hoc analysis (ANOVA) of the BK dose-effect relationship indicated greater total \( R_{mag} \) values (\( P < 0.01 \)) for proestrous units after injection of low doses (0.1 μM) of BK (425 ± 82 spikes per stimulus) than males (262 ± 53) or diestrous females (174 ± 33). Similarly, response duration to 0.1 μM BK was longer (\( P < 0.01 \)) for proestrous units (42 ± 4 s) than male (26 ± 3 s) or diestrous units (28 ± 5 s). Since most TMJ units responded to the lowest dose of BK tested, it was not possible to determine if stage of the estrous cycle significantly altered response thresholds to algesic chemicals injected into the TMJ region.

Although various etiologies likely underlie the onset of TMD pain, elevation of pro-inflammatory factors within the joint space may be a common contributing factor (Denucci et al. 1996; Milam and Schmitz 1995; Stohler 1997). Synovial fluid collected from clinical cases and animal preparations after TMJ injury revealed increased levels of BK, serotonin, substance P, and cytokines (Kopp 1998; Kubota et al. 1998; Swift et al. 1998). Sex steroids exert significant influence on inflammation and immune function (see McKay and Cidlowski 1999). Correspondingly, TMD patients display altered pressure pain thresholds for masticatory muscles (Isselée et al. 2002) and pain severity in relation to positional changes in TMJ disk attachment, an index of TMJ inflammation that varies over the menstrual cycle (Saenaga et al. 2001). These results support
the use of an inflammatory model for TMJ injury and the assessment of reproductive status on processing nociceptive signals from deep craniofacial tissues.

Sex steroids, principally estrogen, have been implicated as causative factors underlying the influence of reproductive status on nociceptive processing and could act at peripheral or central loci. Evidence to support peripheral estrogen-dependent actions include the following: enlarged tactile RF areas of single trigeminal ganglion neurons during estrogen-evoked behavioral estrus (Bereiter and Barker 1980); expression of estrogen receptor-alpha in dorsal root ganglion (Sohrabji et al. 1994) and trigeminal ganglion neurons (this laboratory, unpublished observations); and increased glutamate-evoked TMJ afferent activity in female compared with male rats (Cairns et al. 2001). Also, a high percentage of TMJ primary afferents from male rats were excited by BK; however, female subjects were not included in that study (Takeuchi et al. 2001). Although estrogen levels have been linked to peripheral inflammation (see Bjorling and Wang 2001), it is not clear if differences in neural activity between naïve and inflamed animals share mechanisms with those seen between proestrous and diestrous females. For example, ovariectomy normalized the sex differences in BK-evoked plasma extravasation in the knee joint and estrogen replacement returned these values to those of normal female rats (Green et al. 1999). Administration of exogenous estrogen for 2 weeks increased the expression of the BK B2-receptor in other tissues (Madeddu et al. 1997). By contrast, neither RF area nor thresholds for primary afferent neurons supplying the knee joint were altered significantly by prior inflammation (Dowd et al. 1998; Messinger et al. 1994). Similarly, RF areas of primary afferent neurons that innervate the skin were not different between naïve and inflamed rats, while RF areas of dorsal horn neurons were enlarged after inflammation (Hylden et al. 1989). Estrogen receptor expression in the TMJ disc was similar in symptomatic and asymptomatic TMD female and male patients (Abubaker et al. 1993). Together these results argue against a peripheral mechanism to explain the differences in response properties of TMJ units at the Vc/C2 junction in proestrous and diestrous females.

The signs and symptoms of TMD pain are most consistent with a central neural dysfunction since peripheral pathology of the joint region often is not apparent. Evidence to support central neural mechanisms in TMD pain include the following: spread of pain sensation from the TMJ to adjacent regions of the face (Turp et al. 1998); autonomic and endocrine disturbances (Jones et al. 1997; Korszun et al. 2002); lower thresholds and tolerance for thermal and ischemic pain stimuli applied to other body regions (Maixner et al. 1995); enhanced temporal summation for thermal pain (Maixner et al. 1998); and reduced ability to recruit endogenous pain controls (Bradgon et al. 2002). Also, previous recording studies noted an apparent increase in convergent input onto single brain stem neurons in cats (Rose 1975) and rats (Bradshaw and Berkley 2000) during periods of high circulating estrogen. Several lines of evidence suggest that the Vc/C2 junction region, the initial site of integration for nociceptive signals from the TMJ region, plays a critical role in TMD pain and may be a target for sex hormone modulation. The Vc/C2 junction receives convergent input from all sensory nerves that supply the TMJ region, i.e., trigeminal (Jacquin et al. 1983), upper cervical rootlets (Pflieller and Arvidsson 1988), and vagus (McNeill et al. 1991). A high density of Fos-positive neurons are produced at the superficial laminae at the Vc/C2 junction after acute injury to the TMJ region, proportional to stimulus intensity, and in greater numbers among proestrous than diestrous females or males (Bereiter 2001). The superficial laminae near the Vc/C2 junction region express a high density of estrogen receptor-positive neurons (Amandusson et al. 1996; this laboratory, unpublished observations). The present results further suggested that factors related to stage of the estrous cycle preferentially targeted WDR-like TMJ units. Cutaneous high-threshold RF areas for WDR units were enlarged in proestrous compared with diestrous units, while RF areas for NS units were similar. Iwata et al. (1999) also reported an increase in high-threshold RF areas of WDR neurons in medullary dorsal horn 24 h after inflammation of the TMJ region compared with naïve animals. However, in that study most WDR units were recorded from deeper laminae and only male rats were included. The difference in BK-evoked total Rmag for proestrous and diestrous units was greatest for WDR units. Also, response duration of WDR units after high doses of BK (10 μM) was greater for proestrous than diestrous units, while among proestrous units alone response duration for WDR units was greater than for NS units (see Fig. 8). Although these results suggested a prominent role for WDR-like units, NS units also likely contributed to the processing of chemical inflammatory input from the TMJ region since NS units from both female groups encoded BK concentration. Furthermore, in acute trigeminal recording studies, inflammation of the tooth pulp cavity (Chiang et al. 1998) or deep masseter muscle (Hu et al. 1992) increased the RF area of NS as well as WDR units in subnuclear caudalis of male rats 1–2 h after injury. It cannot be excluded that factors related to the estrous cycle led to apparent phenotypic changes and a shift in the percentage of TMJ units classified as WDR and NS based on cutaneous RF properties. Such changes would not be detected by the “between group” design of the present study.

The mechanisms by which sex steroids modify neural systems involve long-term genomic as well as rapid nongenomic actions (McEwen and Alves 1999) and have been well studied in the hippocampus. Long-term effects of estrogen include alteration in neural structure as well as function. Estrogen administration for 2 days increased dendritic spine density and synaptic connectivity in the hippocampal CA1 region similar to the changes observed over the estrous cycle (Woolley and McEwen 1992; Yankova et al. 2001). Estrogen may act directly through N-methyl-D-aspartate (NMDA)-dependent neurotransmission (Pozzo-Miller et al. 1999) or indirectly through brain-derived neurotrophic factor and reduced GABAergic neurotransmission (Murphy et al. 1998) to increase spine density, and presumably, to enhance the excitability of hippocampal neurons. Rapid effects of estrogen on hippocampal neurons also have been reported and 10-min exposure to estrogen increased NMDA-dependent LTP in tissue slice preparations (Foy et al. 1999). Inflammation-induced neuroplasticity among trigeminal caudalis neurons has been shown in electrophysiological (Chiang et al. 1998) and c-fos studies (Bereiter and Bereiter 2000) to involve NMDA-dependent mechanisms. To determine if acute estrogen administration was sufficient to alter the response properties of TMJ units, in preliminary studies we monitored BK-evoked activity after injection of estradiol benzoate (20 μg, sc) in ovariectomized rats and noted no significant changes in either Rmag or response duration over
2 h (unpublished observations). More than 2 days of estrogen administration were required to increase the cutaneous low-threshold RF area of single trigeminal ganglion neurons (Bereiter and Barker 1980). It is noteworthy that studies designed to examine the influence of estrogen replacement therapy on pain-related behavior or inflammation (Dina et al. 2001; Green et al. 1999; Khasar et al. 2001) have reported significant effects only after several days of treatment. Collectively, these findings are most consistent with the view that sex steroids modulate pain-related neural circuits to affect behavior by longer term genomic rather than rapid nongenomic mechanisms.

Although existing evidence suggests that the Vc/C2 junction is a critical relay for nociceptive input from the TMJ region, it cannot be excluded that other regions of the trigeminal brain stem complex also play a role in processing deep craniofacial pain. Fos-positive neurons are produced bilaterally at the ventrolateral pole of the subnucleus interpolaris/caudalis (Vl/Vc) transition region after acute TMJ injury (Bereiter and Bereiter 2000; Hathaway et al. 1995). However, the number of cells produced at the Vl/Vc transition is not proportional to TMJ stimulus intensity and is similar in females and males (Bereiter 2001). TMJ units also have been recorded in rostral portions of Vc, mainly in deep laminae, from cats of either sex and were excited by different classes of algic chemicals including BK (Broton et al. 1988). Kojima (1990) used electrical and thermal stimuli and found that 80% of TMJ units throughout laminae I–V of Vc in female rats received convergent input from both the TMJ capsule and the masseter muscle; however, chemical stimuli were not tested and the stage of the estrous cycle was not reported.

These results suggested that factors related to the stage of the estrous cycle, rather than sex differences per se, contribute to changes in excitability of TMJ units in laminae I–II at the Vc/C2 junction region. Furthermore, these results indicated that neurons that integrate deep craniofacial input, located in the superficial laminae Vc/C2 junction region, demonstrate considerable capacity for neuroplastic change. Based on cutaneous RF properties and responsiveness to local injection of proinflammatory agents into the TMJ region, the present results were consistent with the hypothesis that central neural mechanisms contribute to enhanced activity by TMJ units during proestrus, the stage of the estrous cycle when estrogen levels are elevated.

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