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NMDA receptor blockade reduces TMJ-evoked activity of trigeminal subnucleus caudalis neurons in an estrogen-dependent manner

A. Tashiro, K. Okamoto, and D.A. Bereiter

Dept. Diagnostic and Biological Sciences, University of Minnesota School of Dentistry, 18-214 Moos Tower, 515 Delaware St. SE, Minneapolis, MN 55455

Abstract

Estrogen status is a risk factor in painful temporomandibular disorders (TMJD). Previously we reported that estradiol (E2) enhanced nociceptive processing of TMJ input by neurons in superficial laminae at the spinomedullary (Vc/C₁₋₂) region; however, the mechanisms for this enhancement are not known. The present study determined if ionotropic glutamate receptors contribute to TMJ nociceptive processing in an E2-dependent manner. Ovariectomized (OvX) female rats were treated with high E2 (HE2) or low dose E2 (LE2) for 2 days and neural activity was recorded in laminae I-II at the Vc/C₁₋₂ region. TMJ-responsive units were activated by adenosine triphosphate (ATP) injections into the joint space. ATP-evoked unit responses in HE2 rats were reduced significantly by topical application of the N-methyl-D-aspartate receptor antagonist, D(-) -2-amino-5-phosphonopentanoic acid (AP5) in a dose-related manner, while units from LE2 were not affected. Application of the non-NMDA receptor antagonist, 6,7-dinitroquinoxaline-2,3-dione (DNQX), inhibited the ATP-evoked responses in both groups. Spontaneous activity of TMJ units was not influenced by AP5, whereas it was reduced by DNQX similarly in both groups. The high threshold convergent cutaneous receptive field area of TMJ units was not changed by AP5, whereas DNQX caused a significant reduction in both groups. These results suggest that NMDA-dependent mechanisms contribute to the enhanced ATP-evoked responses of TMJ units in superficial laminae at the Vc/C₁₋₂ region under high E2 conditions, while non-NMDA-dependent mechanisms modify the encoding properties of TMJ units independent of E2 status.

Keywords

sex steroids; temporomandibular joint; trigeminal brainstem; glutamate receptor

Temporomandibular joint/muscle disorders (TMJD) represent a heterogeneous group of conditions that cause pain in the temporomandibular joint (TMJ) region and masticatory muscles (Dworkin and LeResche, 1992). A notable feature of persistent TMJD is the higher prevalence in women than men (Huang et al., 2002; LeResche, 1997; Slade et al., 2007). Although the basis for the sex difference in TMJD is not certain, clinical findings suggest that

Correspondence: Present address: Akimasa Tashiro, D.D.S., Ph.D., Dept. of Diagnostic and Biological Sciences, University of Minnesota School of Dentistry, 18-214 Moos Tower, 515 Delaware St. SE, Minneapolis, MN, 55455, Phone: (612) 625-4177; Fax: (612) 626-2651; tashi004@umn.edu.

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estrogen status may play a significant role since pain intensity varies over the menstrual cycle (Suenaga et al., 2001; Isselee et al., 2002; LeResche et al., 2003) and hormone replacement therapy is reported to increase TMJD pain in post-menopausal women (LeResche et al., 1997).

Several aspects of TMJD support the involvement of central neural mechanisms (see Sarlani and Greenspan, 2003). For example, persistent TMJD patients often present with few signs of peripheral pathology (Ohrbach and Dworkin, 1998) and display lower thresholds and greater temporal summation to experimental pain than control subjects (Fillingim et al., 1996; Maixner et al., 1998; Svensson et al., 2001). However, peripheral mechanisms cannot be excluded since injection of glutamate into the TMJ produced greater responses in females than males (Cairns et al. 2001). The TMJ region is supplied by small diameter sensory fibers (Kido et al., 1995; Takeuchi and Toda, 2003; Ioi et al., 2006) that project to the trigeminal subnucleus caudalis/upper cervical cord (Vc/C₁₋₂) junction region (Shigenaga et al., 1986; 1988). Previous studies suggested that biological factors related to the estrous cycle modified nociceptive processing since TMJ-evoked responses of neurons in superficial laminae at the Vc/C₁₋₂ region were enhanced in proestrous compared to diestrous in cycling female rats (Okamoto et al., 2003). Similarly, estradiol (E2) treatment also enhanced TMJ-evoked responses of neurons in superficial laminae at the Vc/C₁₋₂ junction in OvX rats (Tashiro et al., 2007).

N-methyl-D-aspartate (NMDA) and non-NMDA glutamate receptors contributed to nociceptive processing of articular input by second-order spinal dorsal horn neurons (Neugebauer et al., 1993; Schaible et al., 2004) and were necessary for central sensitization (Woolf and Salter, 2000; Ji et al., 2003). In the trigeminal brainstem complex, TMJ injury increased the expression of NMDA receptor subunit 1 (NR1) in trigeminal subnucleus caudalis (Vc) (Wang et al., 2009), while pretreatment with either NMDA or non-NMDA receptor antagonists reduced Fos-like immunoreactive (Fos-LI) neurons at Vc/C₁₋₂ region after acute inflammation of the TMJ region (Bereiter and Bereiter, 2000), masseter muscle (Ro et al., 2004) or ocular surface (Bereiter and Bereiter, 1996). However, these studies used only male animals. Recently, we reported that the non-competitive NMDA receptor antagonist, MK-801, greatly reduced the Fos-LI response at the Vc/C₁₋₂ region after TMJ stimulation under high E2 but not low E2 conditions in ovariectomized (OvX) female rats (Okamoto et al., 2008). Although E2 status alters synaptic structure and function in other brain regions through NMDA-dependent receptor mechanisms (Foy, 2001; McEwen, 2002; Woolley, 1999), less is known concerning the relationship between E2 status and ionotropic glutamate receptors in the trigeminal system. The present study tested the hypothesis that the contributions of NMDA and non-NMDA glutamate receptors to TMJ nociceptive processing by neurons in superficial laminae at the Vc/C₁₋₂ region depended on E2 status in the female rat.

Experimental Procedures

The protocols were approved by the Institutional Animal Care and Use Committee of the University of Minnesota and conformed to the established guidelines set by The National Institutes of Health guide for the care and use of laboratory animals (PHS Law 99-158, revised 2002).

General and endocrine procedures

Age-matched, adult ovariectomized (OvX) female rats ($n = 34$, 288 ± 6 g, Sprague-Dawley, Harlan, Indianapolis, IN) were used. Within 10 days after surgery, OvX rats were given a daily injection of either low dose (LE2, 2 μ g, sc, $n = 16$) or high dose (HE2, 20 μ g, sc, $n = 18$) 17 β -estradiol-3-benzoate (E2, Sigma, St. Louis, MO) dissolved in 200 μ l sesame oil for 2 days prior to the experiment. The LE2 and HE2 replacement regimens were chosen to mimic the plasma levels of E2 in diestrus and proestrus, respectively (Smith et al., 1975). The estrogen status of

OvX rats was determined on the day of the experiment by the vaginal smear cytology taken by gentle lavage. Vaginal smears from LE2 rats had (> 80%) small nucleated leukocytes, while smears from HE2 rats had mainly large nucleated epithelial cells or a combination of nucleated and squamous epithelial cells (Montes and Luque, 1988). Vaginal smear results were used to confirm the biological responsiveness of each OvX rat to exogenous E2 treatment. At the end of each experiment an arterial blood sample was collected and plasma levels of E2 were determined by radioimmunoassay (Coat-A-Count, DPC, Los Angeles, CA). Data were collected without prior knowledge of the E2 treatment. The average plasma concentrations for HE2 (n = 17) and LE2 (n = 16) groups were 63.1 ± 13.2 and 8.7 ± 1.6 pg/ml, respectively.

Animal preparation-electrophysiology

Rats were anesthetized initially with pentobarbital sodium (70 mg/kg, ip) and after tracheotomy, were respired artificially with oxygen-enriched room air. Vascular catheters were positioned in the right femoral artery (blood pressure) and right jugular vein (anesthesia). Anesthesia was maintained by a continuous infusion of thiopental sodium (20–30 mg/kg/h) and switched to a mixture of thiopental and a paralytic agent, gallamine triethiodide (15–20 mg/kg/h), after completion of all surgical procedures and just prior to the recording session. Adequate depth of anesthesia was confirmed by the absence of corneal and hind limb withdrawal reflexes prior to gallamine, fully constricted pupils, and constant arterial blood pressure and heart rate. Expiratory end-tidal CO₂ (3.5–4.5%) and mean arterial pressure (MAP, 90–120 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 brainstem surface was bathed in warm mineral oil after surgery. The left temporalis muscle was gently reflected to expose the external pterygoid muscle and the connective tissue overlying 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. A tangential approach (~43° off vertical, 60° off midline) was used to record single units extracellularly with tungsten microelectrodes (9 Mohm, Frederick Haer Inc., Bowdoinham, ME). Unit activity was amplified, discriminated (model DIS-1, BAK Electronics, Mount Airy, MD), stored and analyzed offline on a Macintosh (Apple G4) computer using a DAQ interface board and LabVIEW software (National Instruments, Austin, TX), as described previously (Hirata et al., 1999). Spike amplitude and shape were monitored on a digital oscilloscope and stored on tape for reconfirmation during offline data analyses.

All units included in this study displayed a vigorous response to mechanical probing of the exposed dorsal surface of the posterior condyle and adjacent muscles (see Okamoto et al., 2003, Fig 1). On the basis of cutaneous receptive field properties TMJ units could be further classified as nociceptive specific (NS) and excited by a “press” (arterial clip, ~20 mm²) or “pinch” stimulus (a shorter and stiffer arterial clip, ~15 mm²) applied to facial skin. Brush of facial skin did not activate cells in this study; however, previously we reported that it effectively activated WDR neurons (Okamoto et al. 2003; Tashiro et al. 2007). When applied to the investigator’s forearm skin the press stimulus produced a mild pain sensation, while the pinch stimulus was painful.

Experimental design

One TMJ-responsive unit was recorded in each experiment. TMJ units were recorded from superficial laminae (< 300µm from penetration of the dorsal surface within 1.5 mm rostral to the level of entrance of the C₂ rootlets. Since an acute angle of penetration was used, the exact

vertical distance from the dorsal brainstem surface could not be determined. However, after histological examination, it was estimated that all recording sites were within 200 μm of the dorsal brainstem surface. After confirming the response to posterior condyle stimulation, the face and neck regions were explored for convergent cutaneous input. The cutaneous receptive field (RF) was then tested with brush, press and pinch stimuli. The high threshold cutaneous RF area of each unit was mapped using a small blunt forceps ($\sim 3 \text{ mm}^2$) onto a standardized series of rat face drawings. After mechanical RF stimulation and mapping, a guide cannula (26 gauge) was inserted into the TMJ joint space ($\sim 3 \text{ mm}$ deep) by a dorsal approach directed at the posterior aspect of the mandibular condyle to allow stimulation of the TMJ by chemical stimuli. Test solutions were delivered from a microsyringe attached by polyethylene tubing to an inner cannula (33 gauge) that protruded $\sim 0.5 \text{ mm}$ from the end of the guide cannula consisted of phosphate buffered saline (PBS, pH 7.4) or adenosine triphosphate (ATP, dissolved in PBS) and injected in 20 μl volumes slowly over 30 s to avoid tachyphylaxis. Drug effects were assessed against the response to repeated intra-TMJ injections of ATP (1 mM) at a dose that causes pain sensation in humans (Hamilton et al., 2000; Mork et al., 2003). Previously, we determined that repeated intra-TMJ injections of ATP delivered at 20 min intervals evoked consistent responses in laminae I–II units (Tashiro et al., 2008). The NMDA receptor antagonist, D(–) -2-amino-5-phosphonopentanoic acid (AP5, 0.1 or 1.0 mM, pH = 7.2; Tocris, Ellisville, MO) or non-NMDA receptor antagonist, 6,7-dinitroquinoxaline-2,3-dione (DNQX, 0.01 or 0.1 mM, pH=6.8; Tocris, Ellisville, MO) were applied topically (30 μl) to the dorsal brainstem surface at the Vc/C₁₋₂ region 10 min prior the ATP test stimulus (Fig 1). The angle of the caudal brainstem provided a natural pool for topically applied drugs. Previously we reported that drugs applied to the Vc/C₁₋₂ surface did not affect unit activity in more rostral regions of Vc, approximately 4–6 mm rostral to the Vc/C₁₋₂ region (Meng et al., 1998).

Drugs

AP5 was dissolved in artificial CSF (150 mM NaCl, 2.6 mM KCl, 1.3 mM CaCl₂, and 1.8 mM MgCl₂). DNQX was prepared as a stock solution (10 mM) in dimethylsulfoxide (DMSO, Sigma, St. Louis, MO) diluted fresh before each experiment to its final concentration in artificial CSF. In pilot studies we found that topical application of solutions containing less than 10% DMSO did not affect ATP-evoked responses of TMJ units.

Data analysis

Neural data were acquired and displayed by LabVIEW as a peristimulus time histogram (PSTH) 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 ATP stimulus. The evoked responses were assessed by calculating the response magnitude (Rmag), determined by subtracting the mean plus 2 times the standard deviation (SD) of background activity from the total spike count for each bin. The total Rmag for a given stimulus was defined as the cumulative sum of spikes over contiguous bins in which the spike count minus the background was a positive value. The total Rmag can be considered as equivalent to the “area under the curve” for each stimulus period. Similarly, the 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 + 2 SD above background activity as described previously (Hirata et al., 1999). Units that failed to show three consecutive bins with positive Rmag values within 100 s after stimulus onset were considered unresponsive to that condition. All units included in this study displayed a total Rmag after ATP that exceeded the response to PBS by > 50% prior to drugs. Total Rmag, response duration and response latency to TMJ injections as well as spontaneous activity and cutaneous RF area were assessed statistically by analysis of variance (ANOVA) corrected for repeated measures and individual comparisons were made by Newman-Keuls after ANOVA. The cutaneous high threshold RF areas for each

unit was digitized and quantified by a planimetric method using NIH Image J software without prior knowledge of E2 treatment and compared by ANOVA. A P-value of <0.05 was candidate significant.

Histology

At the end of the experiment Sudan black dye (20 μ l) was injected into TMJ region through the guide cannula to verify placement in the joint space. The recording site was marked electrolytically (5 μ A, 20 s). The animal was given a bolus dose of thiopental sodium (60 mg/kg, iv) and perfused through the heart with 10% formalin. Recording sites were drawn onto a standard series of rat brainstem outlines (Takeshita et al., 2001).

Results

E2 status and NMDA receptor blockade

A total of 19 ATP-responsive units, classified as nociceptive specific (NS), were recorded in laminae I-II at the Vc/C₁₋₂ region from HE2 (n = 10) and LE2 (n = 9) rats and tested before and after application of AP5 to the dorsal brainstem surface. The depth of recording averaged 199 ± 29 μ m and 182 ± 28 μ m for HE2 and LE2 groups, respectively, and were located as previously reported (Takeshita et al., 2001, Okamoto et al., 2003; 2005). As seen in Fig 1, intra-TMJ injection of ATP evoked a prompt increase in unit activity and the response of units from HE2 rats (Fig 1A) was greater than that from LE2 units (Fig 1B) prior to AP5, consistent with previous results (Tashiro et al., 2007). Topical application of AP5 significantly reduced the ATP-evoked total Rmag in the HE2 group ($F_{3,51} = 8.75$, $P < 0.001$) to a value similar to the LE2 group, while AP5 did not affect the ATP-evoked total Rmag of units in LE2 rats (Fig 2A). In all cases the average ATP-evoked total Rmag returned to near the pre-drug value immediately after washout in the HE2 group. The reduction in total Rmag after high dose AP5 was a consistent finding as 8 of 10 units from HE2 rats displayed reductions of $>25\%$, whereas only 2 of 9 units from LE2 rats had a similar level of reduction. The ATP-evoked response duration from HE2 and LE2 averaged 48 ± 5 and 35 ± 3 s, respectively, before AP5 ($P > 0.05$) and was not changed significantly after AP5, although a small numerical reduction was seen in HE2 units after high dose AP5 (Fig 2B). Spontaneous activity (SA) of TMJ units from HE2 and LE2 rats averaged 4.60 ± 0.96 and 2.09 ± 0.52 spikes/s, respectively, before the initial ATP test stimulus ($P > 0.05$) and was not affected significantly ($P > 0.05$) by AP5 in either group (Fig 2C). The high threshold convergent cutaneous receptive field (RF) area of TMJ units from HE2 and LE2 rats averaged 1.8 ± 0.2 and 1.2 ± 0.2 cm², respectively, before AP5 ($P > 0.05$) and was not changed significantly after high dose AP5 in either group ($< 10\%$ change from pre-drug value, $P > 0.05$). Mean arterial pressure averaged 97 ± 4 mmHg and 96 ± 5 mmHg for HE2 and LE2 females, respectively, prior to drug application and was not changed after AP5 (data not shown).

E2 status and non-NMDA receptor blockade

A total of 14 ATP-responsive units, classified as nociceptive specific (NS), were recorded in laminae I-II at the Vc/C₁₋₂ region from HE2 (n = 7) and LE2 (n = 7) rats and tested before and after application of DNQX to the dorsal brainstem surface. The depth of recording were similar for HE2 and LE2 groups and averaged 224 ± 34 μ m and 183 ± 35 μ m, respectively. Topical application of DNQX caused a rapid and significant reduction in evoked and spontaneous activity of TMJ units in both groups (Fig 3). The ATP-evoked total Rmag of TMJ units from HE2 and LE2 rats was reduced in a dose-related manner ($F_{3,36} = 23.7$, $P < 0.001$) and reversed during the washout period. As summarized in Fig 4A, even low dose DNQX (0.01 mM) was sufficient to significantly reduce the average ATP-evoked total Rmag. The reduction in ATP-evoked total Rmag after DNQX was a consistent finding as all units from HE2 and LE2 rats had reductions of $>25\%$ after high dose DNQX (0.1mM). The ATP-evoked response duration

from HE2 and LE2 rats averaged 38 ± 5 and 39 ± 3 s, respectively, before DNQX ($P > 0.05$) and was reduced significantly by DNQX ($F_{3,36} = 15.4$, $P < 0.001$) in both groups and rapidly returned to pre-drug values after washout (Fig 4B). Spontaneous activity (SA) of TMJ units from HE2 and LE2 rats averaged 4.60 ± 1.07 and 3.50 ± 1.26 spikes/s, respectively, before the initial ATP test stimulus ($P > 0.05$). Local application of DNQX also caused a marked reduction in SA of TMJ units from HE2 and LE2 rats (Fig 4C, $F_{3,36} = 16.2$, $P < 0.001$). The high threshold convergent cutaneous RF area of TMJ units of HE2 and LE2 rats averaged 2.1 ± 0.2 and 1.6 ± 0.2 cm², respectively, before drug treatment ($P > 0.05$) and were reduced significantly ($F_{2,20} = 70.5$, $P < 0.001$) after high dose DNQX in both groups (HE2 = $-47.3 \pm 5\%$; LE2 = $-42.8 \pm 8\%$ versus pre-drug). Mean arterial pressure averaged 104 ± 3 mmHg and 106 ± 4 mmHg for HE2 and LE2 rats, respectively, prior to drug application and was not changed after DNQX (data not shown). Stable resting blood pressure after topical application of AP5 or DNQX at the Vc/C1-2 region suggested that these drugs did not diffuse to autonomic control regions such as the nucleus tractus solitarius.

Discussion

This study found that E2 status selectively modified the responsiveness of TMJ units in superficial laminae at the Vc/C1-2 region to local NMDA receptor blockade such that the normally enhanced TMJ-evoked response during high E2 was reduced to that seen during low E2 conditions. TMJ units from LE2-treated rats were not affected by NMDA receptor blockade. The effect of AP5 on TMJ units was selective for TMJ-evoked activity as neither spontaneous activity or the size of the convergent cutaneous RF was affected. By contrast, non-NMDA receptor blockade by DNQX had widespread effects on the properties of TMJ units and inhibited both evoked and spontaneous activity and reduced the size of the convergent cutaneous RF independent of E2 status.

NMDA receptor activation influences diverse neural functions in an estrogen-dependent manner in the female brain. This notion was strongly supported by studies in hippocampus where exposure to E2 in a pattern similar to that of proestrus rats produced persistent changes in synaptic plasticity and dendritic morphology of CA1 pyramidal neurons through NMDA receptor-dependent mechanisms (see McEwen, 2002; Cooke and Woolley, 2005). In hypothalamus, NMDA receptor activation increased the secretion of luteinizing hormone in the presence of proestrous levels of E2, while minor effects were seen under low E2 conditions (see Brann and Mahesh, 1995). NMDA receptor modulation of sensorimotor integration in the female rat also depended on E2 status since elevated plasma levels of E2 facilitated NMDA-dependent activity in cerebellar Purkinje cells that persisted for hours (Smith, 1989; 1994).

Although it is possible that E2 also acts through peripheral mechanisms to alter Vc/C1-2 neurons, the significance of peripheral glutamate actions in relation to estrogen status remains uncertain. For example, intra-TMJ injection of a high concentration of glutamate (500 mM) evoked greater A-delta TMJ afferent fiber activity from female than male rats; however, responses were similar for females at different stages of the estrous cycle (Cairns et al. 2001b). Similarly, injection of high concentrations of glutamate (500 mM) into masseter muscle evoked greater responses from A-delta muscle afferents from female rats than males, while afferents from females at different stages of the estrous cycle had similar responses (Cairns et al. 2001a). However, it should be noted that in both studies C-fibers were not sampled and the estimated termination zone for these afferents, based on antidromic activation from Vc, was 2–4 mm rostral to the level recorded in the present study. 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). Intra-articular (Ayesh et al., 2008) or masseter muscle (Castrillon et al., 2008) injection of the NMDA receptor antagonist, ketamine, in TMJ patients did not reduce arthralgic pain in women

suggesting that peripheral NMDA receptor activation may play only a minor role in mediating TMJ-related pain in women.

The superficial laminae at the Vc/C₁₋₂ region express a high density of estrogen receptor (ER) positive neurons (Amandusson et al., 1996; Bereiter et al., 2005) and NMDA receptors (Bereiter and Bereiter, 2000; Tang et al., 2001; Wang et al., 2009); however, little is known concerning the relationship between E2 status and NMDA receptor-dependent mechanisms at this initial site for integration of nociceptive inputs from craniofacial tissues. The number of Fos-positive neurons produced after TMJ stimulation at the Vc/C₁₋₂ region was enhanced in HE2- compared to LE2-treated rats, an effect that was prevented by the non-competitive NMDA antagonist, MK801, whereas Fos-LI in LE2-treated rats was not affected, consistent with the present results (Okamoto et al., 2008). Microinjection of NMDA into the caudal trigeminal brainstem produced a similar amount of orofacial scratching behavior in males and OvX female rats that was reduced by co-injection of the adrenergic agonist clonidine, whereas E2-treated OvX rats failed to respond to clonidine (Nag and Mokha, 2006). NMDA-evoked orofacial scratching in males and OvX females was reduced by the opioid receptor-like agonist, nociceptin, while E2-treated OvX females had increased scratching. Nociceptin also inhibited NMDA-evoked activity in caudal Vc laminae I–II units of most cells in males and OvX females, while fewer units were inhibited in E2-treated OvX females (Flores et al., 2001). Nearly 50% of ER- α positive neurons in laminae I–II at the Vc/C₁₋₂ region contained GABA (Bereiter et al., 2007) or enkephalin (Amandusson et al., 1996) consistent with the phenotype of inhibitory interneurons. In spinal dorsal horn 37% of GABAergic neurons in superficial laminae also expressed the NMDA receptor subtype NR1 (Lu et al., 2005). Although exact mechanisms are not yet known, these studies suggest that E2 may act through multiple pathways to alter NMDA-mediated trigeminal nociceptive behavior.

If a low E2 condition per se is a significant factor in dorsal horn nociceptive processing, then the effects of NMDA and non-NMDA receptor antagonists on TMJ units in LE2-treated females and males might be expected to share similar features. Indeed, Luccarini et al. (2001) used male rats and found that windup in lamina I Vc units evoked by percutaneous electrical stimulation of facial skin was not affected by dorsal horn application of NMDA. Seagrove et al. (2004) also used male rats and electrical stimulation of the hind limb to activate lamina I units in lumbar dorsal horn and found that AP5 applied to the spinal cord surface did not alter C-fiber evoked responses or wind-up, whereas the non-NMDA antagonist, NBQX, inhibited A-delta and C-fiber evoked responses as well as wind-up. It was possible that our failure to observe AP5-induced inhibition of TMJ units in LE2 rats was due to methodological differences such as drug dosage or the modality of the applied TMJ stimulus i.e., ATP microinjection, however, this seems unlikely. The doses of AP5 used (high dose = 30 nmol or ~6 μ g) were similar to those shown to block visceromotor reflexes in OvX female rats (Tang et al., 2008), phase 2 responses in the formalin test in male rats (Chaplan et al., 1997) and mustard oil-induced hyperalgesia in spinalized male rats (Silva et al., 1997). Since intra-TMJ microinjections of ATP were used to drive neural activity, it was possible that the modality of this stimulus influenced the effectiveness of AP5 in an E2-dependent manner by selecting for subpopulations of sensory fibers. However, ~50% of TMJ afferents express P2x receptors (Shinoda et al., 2005), and in spinal cord studies ATP activated most A-delta and C-fiber afferents from the knee joint (Dowd et al., 1998) and spinal lamina I cells receiving small fiber input (Chen and Gu, 2005). These studies suggested that P2x receptors have a significant role in nociceptive processing and that the ATP stimulus did not select for a narrow subpopulation of small diameter TMJ afferents. The present study tested drug effects on TMJ unit activity in naïve rats only, thus it could not be excluded that ongoing inflammation would have increased the likelihood of observing an NMDA antagonism in LE2-treated rats. For example, intrathecal injection of AP5 did not affect thermal nociceptive behavior in naïve male rats, whereas it was greatly reduced behavior during inflammation (Ren et al., 1992).

Although AP5 reduced the ATP-evoked unit responses of HE2 rats, it did not inhibit evoked responses below that seen for LE2 units. By contrast, DNQX caused a nearly complete inhibition of ATP-evoked and spontaneous activity of TMJ units in HE2 and LE2 groups. Thus, a second possible indirect mechanism to explain differential effects of NMDA receptor blockade on TMJ-evoked activity under HE2 versus LE2 conditions may involve the ratio of NMDA and AMPA function. In hippocampus E2-induced changes in excitability depended on a tight mechanistic coupling of NMDA and AMPA receptor transmission, a coupling that would be altered under high E2 conditions due to upregulation of NMDA receptors (Smith and McMahon, 2005). Since the vast majority of spinal cord superficial laminae neurons that contained NMDA receptors also expressed the non-NMDA receptor subtype GluR2 (Nagy et al., 2004b; Antal et al., 2008), high E2 conditions leading to increased NMDA receptor expression in dorsal horn (Tang et al., 2008) would be expected to increase the NMDA:non-NMDA current ratio and thus favoring increased neuronal excitability. An altered ratio of NMDA to non-NMDA current in spinal dorsal horn has been proposed as a mechanism for hyperalgesia and allodynia following nerve injury (Iwata et al., 2007).

The effects of topical application of drugs to the dorsal brainstem surface could have resulted from actions at receptors on presynaptic axon terminals of primary afferents or postsynaptic second-order neurons (Nagy et al., 2004a; Liu et al., 1994; Lu et al., 2003). Activation of presynaptic NMDA receptors resulted in reduced glutamate release in an in vitro preparation (Bardoni et al., 2004); however, only minor effects on neuropeptide release in dorsal horn were seen in vivo (Nazarian et al., 2008).

In conclusion, enhanced TMJ-evoked responses of superficial laminae neurons during high E2 conditions were due, at least in part, to increased NMDA receptor activity. The interaction between E2 status and NMDA receptor activity likely involved stimulus-specific mechanisms since neither background activity nor convergent receptive field size of TMJ units was modified by AP5. By contrast, non-NMDA mechanisms had widespread effects on the properties of TMJ units independent of E2 status. The present data suggest that ionotropic glutamate receptors play critical role in TMJ nociceptive processing and likely contribute to pain sensation in TMJD in women.

Acknowledgments

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Abbreviations

TMJ	temporomandibular joint
Vc/C ₁₋₂	trigeminal subnucleus caudalis/upper cervical cord
E2	estradiol
OvX	ovariectomized
PBS	phosphate buffered saline
ATP	adenosine triphosphate
NMDA	N-methyl-D-aspartate receptor
AP5	D(-) -2-amino-5-phosphonopentanoic acid
DNQX	6,7-dinitroquinoxaline-2,3-dione
DMSO	dimethylsulfoxide

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Figure 1.

Peristimulus time histograms displaying examples of the effects of the NMDA receptor antagonist, AP5, on ATP-evoked responses of TMJ units from (A) HE2 and (B) LE2 rats. Abbreviations: ATP (intra-TMJ injection, 1 mM, 20 μ l); 1 bin = 1 s. Arrows refer to time of drug application (AP5; 0.1, 1mM), 10 min before the ATP injection.

Figure 2.

Effect of AP5 on total Rmag (A), response duration (B) and spontaneous activity (C) of TMJ units recorded in superficial laminae of the Vc/C₁₋₂ region. Sample sizes: HE2, n = 10; LE2, n = 9. **P < 0.01 versus ATP alone; a = P < 0.05, b = P < 0.01 versus LE2 group.

Figure 3.

Peristimulus time histograms displaying examples of the effects of DNQX on ATP-evoked responses of TMJ units classified as NS units from (A) HE2 and (B) LE2 rats. Arrows refer to time of drug application (DNQX; 0.01, 0.1 mM), 10 min before the ATP injection.

Figure 4. Effect of DNQX on total Rmag (A), response duration (B) and spontaneous activity (C) of TMJ units recorded in superficial laminae of the Vc/C₁₋₂ region. Sample sizes: HE2, n = 7; LE2, n = 7. *P < 0.05, **P < 0.01 versus ATP alone; a = P < 0.05 versus LE2 group.