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GABAergic influence on temporomandibular joint-responsive spinomedullary neurons depends on estrogen status

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Abstract

Sensory input from the temporomandibular joint (TMJ) to neurons in superficial laminae at the spinomedullary (Vc/C₁₋₂) region is strongly influenced by estrogen status. This study determined if GABAergic mechanisms play a role in estrogen modulation of TMJ nociceptive processing in ovariectomized female rats treated with high (HE) or low dose (LE) estradiol (E2) for two days. Superficial laminae neurons were activated by ATP (1 mM) injections into the joint space. The selective GABA_A receptor antagonist, bicuculline methiodide (BMI, 5 or 50 μM, 30 μl), applied at the site of recording greatly enhanced the magnitude and duration of ATP-evoked responses in LE rats, but not in units from HE rats. The convergent cutaneous receptive field (RF) area of TMJ neurons was enlarged after BMI in LE but not HE rats, while resting discharge rates were increased after BMI independent of estrogen status. By contrast, the selective GABA_A receptor agonist, muscimol (50 μM, 30 μl), significantly reduced the magnitude and duration of ATP-evoked activity, resting discharge rate, and cutaneous RF area of TMJ neurons in LE and HE rats, whereas lower doses (5 μM) affected only units from LE rats. Protein levels of GABA_A receptor β3 isoform at the Vc/C₁₋₂ region were similar for HE and LE rats. These results suggest that GABAergic mechanisms contribute significantly to background discharge rates and TMJ-evoked input to superficial laminae neurons at the Vc/C₁₋₂ region. Estrogen status may gate the magnitude of GABAergic influence on TMJ neurons at the earliest stages of nociceptive processing at the spinomedullary region.

Introduction

Temporomandibular joint/muscle disorders (TMJD) represent a heterogeneous, often idiopathic, group of pain conditions involving the temporomandibular joint (TMJ) region

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and masticatory muscles (Dworkin and LeResche, 1992; Maixner 2009; Bereiter and Okamoto, 2011). A prominent feature of persistent TMJD is the higher prevalence in females than in males (Huang et al., 2002; LeResche, 1997; Slade et al., 2007). Although the etiology of TMJD is not known, considerable evidence suggests that estrogen status plays a significant role. Pain intensity varies over the menstrual cycle (Isselee et al., 2002; LeResche et al., 2003; Landi et al., 2005), hormone replacement therapy increases jaw pain in postmenopausal women (LeResche et al., 1997), and genetic polymorphisms of estrogen receptors (ER) affect the susceptibility to develop TMJD (Ribiero-DaSilva et al. 2009).

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 superficial laminae at the Vc/C₁₋₂ region (Shigenaga et al., 1986; 1988). The superficial laminae at the Vc/C₁₋₂ region share many properties with corresponding regions at lower segments of the spinal cord (Bereiter et al., 2000) and receive the majority of input from unmyelinated sensory fibers (Kobayashi and Matsumura, 1996; Sugimoto et al., 1997). Mechanisms of central sensitization and disinhibition of inhibitory signals within the superficial laminae are thought to be critical for the development of chronic pain (see Woolf and Salter 2000; Suzuki et al. 2002; Todd 2010). Previously we determined that the response properties of TMJ neurons in superficial laminae varied significantly over different stages of the estrous cycle in intact female rats (Okamoto et al. 2003), while exogenous E2 treatment increased the TMJ-evoked responses of neurons in superficial but not in deeper laminae in ovariectomized female rats (Tashiro et al. 2007; Okamoto et al. 2013). Although estrogen status markedly affected the response of TMJ-responsive neurons to NMDA receptor antagonism (Tashiro et al., 2009a) and inhibition of MAP kinase activity consistent with central sensitization (Tashiro et al., 2009b), little is known about estrogen status, disinhibition and GABAergic function in TMJ nociception. GABAergic neurons are found throughout the trigeminal brainstem sensory complex and are densely distributed in superficial laminae of Vc (Ginestal and Matute, 1993; Polgar and Antal, 1995; Avendano et al., 2005). Estrogen receptor (ER)-positive neurons also are found in superficial laminae of Vc and colocalize preproenkephalin mRNA (Amandusson et al., 1996) or GABA (Bereiter et al., 2005; 2007). GABA acts through ionotropic GABA_A receptors as well as G protein-coupled GABA_B receptors to alter sensory processing at spinal levels (Malcangio and Bowery, 1996; Hammond, 1997). Estrogen status influences GABA biosynthesis by targeting the gad2 promoter (Hudgens et al. 2009) and, more indirectly, by altering the expression of GABA_A receptor subunits in the trigeminal ganglion (Puri et al. 2011) and elsewhere in the brain (McCarthy et al. 1995; Nakamura et al. 2004; Lovick 2008). To determine if estrogen status alters GABAergic influences on TMJ nociception, we recorded from Vc/C₁₋₂ neurons under high (HE) and low (LE) estrogen conditions in ovariectomized female rats and tested the effects of locally applied antagonists and agonists for GABA_A receptors. To assess possible trophic effects of E2 on GABA_A receptors we also measured $\beta 3$ subunit isoform levels by western blot. The $\beta 3$ isoform is found in more than 80% of all GABA_A receptors in the brain (Benke et al. 1994) and is well distributed in spinal dorsal horn of the rat (Alvarez et al. 1996).

Experimental Procedures

Study protocols were approved by the Committee of Research Facilities for Laboratory Animal Science, National Defense Medical College (Japan) and the Institutional Animal Care and Use Committee of the University of Minnesota. The protocols 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 Sprague–Dawley rats weighing 250–320 g (SLC, Shizuoka, Japan; Sprague–Dawley, Harlan, Indianapolis, IN) were used. Within 14 days after surgery, OvX rats were administered a daily injection of either low dose (LE, 2 µg, s.c.) or high dose (HE, 20 µg, s.c.) 17α-estradiol-3-benzoate (E2, Sigma, St. Louis, MO) dissolved in 200 µl sesame oil for two days before the experiment. The LE and HE replacement regimens were selected 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 vaginal smear cytology obtained by gentle lavage. Vaginal smears from the LE rats contained >80% small nucleated leukocytes, whereas smears from the HE rats primarily consisted of large nucleated epithelial cells or a combination of nucleated and squamous epithelial cells (Montes and Luque, 1988). Data were collected without prior knowledge of the E2 treatment. Plasma E2 levels were not routinely measured; however in a previous study using a similar dosing regimen we found that low E2 and high E2 treated OvX females had <20 and 50–100 pg/ml, respectively (Tashiro et al 2009a).

Animal preparation: electrophysiology

Rats were anesthetized initially with pentobarbital sodium (50 mg/kg, i.p.), and catheters were positioned in the right femoral artery and jugular vein for monitoring blood pressure and drug infusion, respectively. After tracheotomy, animals were respired artificially with oxygen-enriched room air and anesthesia was maintained with isoflurane (1.0%–1.5%). Rats received an infusion of the short-acting paralytic agent, gallamine triethiodide (25 mg/kg/h) at the time of neural recording. Expiratory end-tidal CO₂ (3.5%–4.5%), mean arterial pressure (MAP, 100–120 mm Hg), and body temperature (38°C) were monitored continuously and maintained within normal range.

Animals were placed in a stereotaxic frame and dorsal 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 exposing 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, 4–7 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 (5 MOhm, Epoxylite coated, tip diameter <1 µm, Bio Research Center Co. Nagoya, Japan). Unit activity was amplified, discriminated (model WD-2, Bio Research Center Co., Nagoya, Japan), stored,

and analyzed offline using a PowerLab interface and LabChart software (ADInstruments, Bella Vista, Australia).

All units included in this study displayed a vigorous response to gentle mechanical probing of the exposed dorsal surface of the condyle and adjacent muscles. All TMJ units were classified as nociceptive specific (NS) and excited by a “press” (arterial clip, approximately 20 mm²) or “pinch” stimulus (shorter and stiffer arterial clip, approximately 15 mm²) applied to facial skin but not to brushing. When applied to the investigator’s forearm skin, the press stimulus produced mild pain sensation, whereas the pinch stimulus was considerably painful.

Experimental design

Beginning at least 2h after initial anesthesia, TMJ units were recorded from superficial laminae <200 μ m of the dorsal surface within 1.5 mm rostral to the level of the entrance of the C₂ rootlets as determined by histological examination of recovered recording sites. Prior to recording, depth of anesthesia was similar for HE and LE rats as determined by the loss of corneal and hindpaw withdrawal reflexes. One TMJ-responsive unit was recorded in each experiment. After confirming the response to posterior condyle stimulation, the face and neck were explored for convergent cutaneous input. The high threshold cutaneous RF area of each TMJ unit was mapped using a small blunt forceps (~3 mm²) 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 (approximately 3 mm deep) by a dorsal approach directed toward the posterior aspect of the mandibular condyle to allow repeated delivery of chemical stimuli. Test solutions were injected from a microsyringe attached by polyethylene tubing to an inner cannula (33 gauge) that protruded approximately 0.5 mm from the end of the guide cannula. Test solutions consisted of phosphate buffered saline (PBS, pH 7.4) or adenosine triphosphate (ATP, 1 mM, in PBS), which were slowly injected in 20 μ l aliquots over 30 s to avoid tachyphylaxis. This dose of ATP (1 mM) is reported to evoke pain sensation in humans (Hamilton et al., 2000; Mork et al., 2003). Previously, we determined that repeated intra-TMJ injections of 1 mM ATP delivered at 20-min intervals evoked consistent responses in Vc/C₁₋₂ neurons (Tashiro et al., 2008). Mineral oil was removed before topical drug application. The selective GABA_A receptor antagonist, bicuculline methiodide (BMI, 5 or 50 μ M, Tocris, Ellisville, MO, in aCSF), or the GABA_A receptor agonist, muscimol (5 or 50 μ M, Tocris, in aCSF), was topically applied (30 μ l) at the site of recording 10 min prior to the ATP test stimulus. The doses applied were consistent with those shown to be effective on lumbar dorsal horn (Zhang et al. 2001). 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 (Meng et al. 1998)

Data analysis

Neural data were acquired and displayed by LabChart as a peristimulus time histogram (PSTH) of spikes/s (1 s bins), exported to a spreadsheet and analyzed offline. Spontaneous activity (SA, spikes/s) was calculated as the average spike count over a 1-min period immediately preceding each ATP stimulus. Drug effects on SA were determined from the

average spike counts over 1 min prior to and for the first minute after administration. The evoked responses were assessed by calculating the response magnitude (Rmag), determined by subtracting the mean plus two 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. Response duration was defined as the time interval after stimulus onset until three consecutive bins with a positive spike count occurring above the background (initial latency), and until the value of three consecutive bins no longer exceeded the mean + 2SD above the background activity. Response latency was defined as the earliest time after stimulus onset for which three consecutive 1 s bins exceeded the background activity (i.e., Rmag). 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%. The cutaneous high threshold RF area for each unit was digitized and quantified using a planimetric method and NIH ImageJ software without prior knowledge of E2 treatment. Total Rmag, response duration, and latency to TMJ stimulation as well as spontaneous activity and cutaneous RF area were statistically assessed using analysis of variance (ANOVA) corrected for repeated measures, and individual comparisons were made using the Newman–Keuls test after ANOVA. A *P* value of < 0.05 was considered statistically significant.

Histology

The recording site was marked electrolytically (5 μ A, 20 s). The animal was administered a bolus dose of pentobarbital sodium (60 mg/kg, i.v.) and perfused through the heart with 10% formalin. Recovered lesion sites were mapped onto a standard series of rat brainstem outlines.

GABA_A receptor western blots

The Vc/C₁₋₂ region (3 to 6 mm caudal to the obex) was removed from LE and HE rats (250–300 g; *n* = 6/group) after saline perfusion and homogenized in 0.5 mL cold lysis buffer [1% Triton X-100, 10 mM EGTA, 10 mM EDTA, TBS; pH 7.4; with ease inhibitor cocktail (Complete Mini, Roche, Indianapolis, IN)]. The homogenates were centrifuged at 4°C for 10 min at 12,000 $\times$ g, and the supernatant was retained. Protein concentration was determined by BCA assay (Pierce, Rockford, IL), and 25 μ g protein was separated on 7.5% polyacrylamide gels and transferred to a 0.45 μ M nitrocellulose membrane (BioRad, Hercules, CA). Membranes were blocked and incubated at 4°C overnight in GABA_A β 3 receptor antibody (AB5563, Millipore, Billerica, MA) at 1 μ g/mL, followed by goat anti-rabbit IRDye 680 (LI-COR, Lincoln, NE). This antibody was cloned from rat brain and characterized previously (Slany et al. 1995). Proteins were visualized using an Odyssey infrared scanner (LI-COR) and arbitrary optical density was determined. Normalizing controls were utilized by simultaneous staining with beta-tubulin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) followed by goat anti-mouse IRDye 800 secondary antibody (LI-COR).

Results

E2 status and GABA_A receptor antagonism by bicuculline

A total of 12 ATP-responsive units were recorded in superficial laminae at the Vc/C₁₋₂ region from the HE (n = 6) and LE (n = 6) rats and tested before and after local application of BMI to the dorsal brainstem surface. As shown in the examples of Fig 1, prior to BMI application, intra-TMJ injections of ATP evoked a prompt increase in activity in HE units (Fig. 1A) that was greater than that in LE units (Fig 1B) ($F_{1,17} = 6.57$, $P < 0.05$), consistent with our previous studies (Tashiro et al., 2007). BMI significantly enhanced the ATP-evoked Rmag in LE rats ($F_{3,30} = 10.5$, $P < 0.001$), whereas the average Rmag for units in HE rats was not affected (Fig. 2A). This was a consistent finding as four of six units from LE rats displayed ATP-evoked Rmag values that exceeded that of the pre-BMI value by >50%, whereas none of the six units from HE rats were enhanced. Prior to BMI, the ATP-evoked response duration also was significantly longer ($F_{1,30} = 10.5$, $P < 0.005$) in HE units compared to LE units (Fig. 2B), whereas after BMI the ATP-evoked response duration was significantly prolonged in LE units but not in HE units (Fig. 2B, $F_{3,30} = 5.27$, $P < 0.025$). The ATP-evoked response latencies were similar for LE and HE units (12.8 ± 2.1 and 9.2 ± 1.8 s, respectively) prior to BMI and did not change significantly after drug application (Fig 2C). The SA of TMJ units from HE and LE rats averaged 3.52 ± 0.54 spikes/s and 1.42 ± 0.58 spikes/s, respectively, before drug administration and BMI (50 μ M) caused a significant increase in SA ($F_{1,10} = 7.50$, $P < 0.025$) in both groups (HE = 6.9 ± 2.2 , LE = 3.5 ± 0.8 spikes/s). The high threshold convergent cutaneous receptive field (RF) area of TMJ units in HE and LE rats averaged 1.4 ± 0.2 cm² and 1.3 ± 0.2 cm², respectively, before drug treatment. The RF area was re-measured after 50 μ M BMI and found to be enlarged by nearly 20% in LE units ($F_{1,10} = 5.04$, $P < 0.05$), while the RF area of HE units was not changed. Mean arterial pressure averaged 112 ± 8 mmHg and 108 ± 4 mmHg for the HE and LE rats, respectively, before drug application and did not change after BMI application (data not shown).

E2 status and GABA_A receptor activation by muscimol

A total of 14 ATP-responsive units were recorded in superficial laminae at the Vc/C₁₋₂ region from the HE (n = 7) and LE (n = 7) rats and tested before and after local application of muscimol to the dorsal brainstem surface. As shown in Figure 3, prior to muscimol the ATP-evoked response of HE units (Fig 3A) was greater than that seen in LE units (Fig. 3B, $F_{1,33} = 10.7$, $P < 0.005$). As seen in Fig 4A, low dose muscimol (5 μ M) reduced the ATP-evoked Rmag in HE units ($F_{3,36} = 20.1$, $P < 0.001$) to that seen in LE units. The reduction in total Rmag after low-dose muscimol in HE units was a consistent finding as six of seven units displayed reductions of >50%, whereas only one of seven LE units displayed a similar level of reduction. However, high dose muscimol (50 μ M) greatly reduced the total Rmag of both HE and LE units ($F_{3,36} = 4.45$, $P < 0.025$) consistent with the notion of ongoing high GABAergic tone under LE conditions. The ATP-evoked response duration was significantly longer ($F_{1,45} = 11.2$, $P < 0.005$) in HE units than in LE units prior to muscimol (Fig 4B). Low dose muscimol also reduced the ATP-evoked response duration in HE units to the value seen in LE units ($F_{3,36} = 25.8$, $P < 0.001$); however, high dose muscimol greatly reduced the evoked response duration in both LE and HE units ($F_{3,36} = 15.6$, $P < 0.001$).

ATP-evoked response latencies were similar for HE and LE units (7.1 ± 1.3 s and 11.6 ± 2.7 , respectively) prior to muscimol and increased significantly and similarly in HE and LE units after muscimol ($F_{3,36} = 21.7$, $P < 0.001$). The SA in HE and LE units averaged 2.77 ± 0.96 and 3.12 ± 0.93 spikes/s, respectively, before muscimol. Low dose muscimol did not affect SA, whereas high dose muscimol caused a significant and similar reduction in HE and LE units ($F_{3,36} = 8.7$, $P < 0.001$). The high threshold convergent cutaneous RF area of TMJ units in HE and LE rats averaged 1.4 ± 0.2 cm² and 1.1 ± 0.1 cm², respectively, before muscimol. High dose muscimol caused a marked and similar decrease ($F_{1,12} = 69.3$, $P < 0.001$) in RF area of HE ($52.3 \pm 10.8\%$ vs pre-drug) and LE units ($47.8 \pm 7.7\%$ vs pre-drug). Mean arterial pressure averaged 106 ± 2 mmHg and 108 ± 3 mmHg for the HE and LE rats, respectively, before drug administration and was not altered by topical muscimol (data not shown).

GABA_A receptor Western blots

Western blot analysis was used to determine the GABA_A $\beta 3$ receptor protein levels from additional groups of HE and LE female rats. In caudal Vc tissue samples that included the Vc/C₁₋₂ region, E2 treatment did not affect GABA_A $\beta 3$ receptor protein levels (mean relative OD; HE = 3.22 ± 1.26 , LE = 4.3 ± 1.29).

Discussion

This study demonstrated for the first time that resting discharge rates and TMJ-evoked responses of superficial laminae neurons at the Vc/C₁₋₂ region were significantly modified by GABA_A receptor activity that was, in turn, dependent on estrogen status. Under HE conditions disinhibition of GABA_A receptors at the Vc/C₁₋₂ region by the selective antagonist, BMI, increased resting discharge rates, but did not affect the magnitude of TMJ-evoked activity or the size of the convergent cutaneous RF on TMJ units. Conversely, activation of GABA_A receptors by muscimol under HE conditions markedly decreased the resting discharge rates, TMJ-evoked activity and the size of the convergent cutaneous RF. Under LE conditions, BMI increased resting discharge rates, TMJ-evoked activity and the size of the cutaneous RF, while muscimol decreased discharge rates, TMJ-evoked activity and the cutaneous RF area. One interpretation of these results was: a) compared to LE conditions, HE is associated with a higher state of neuronal excitability and lowered GABAergic tone such that no further enhancement of TMJ-evoked responses is possible after local BMI, b) activation of GABA_A receptors by exogenous muscimol inhibits resting and evoked activity of TMJ units and, at sufficient doses, can override even the high GABAergic tone of LE rats and c) GABAergic modulation of background discharge rates and TMJ-evoked responses rely on separate, though not mutually exclusive pathways. A dual pathway for control of resting and evoked activity in TMJ-responsive lamina I neurons is represented in Fig 5. As reviewed by Todd (2010), the majority of nociceptive primary afferent fibers projecting to superficial laminae terminate on excitatory and inhibitory interneurons, many of which are GABAergic. However, GABA input to dorsal horn neurons also derives from descending projections in RVM. GABAergic modulation of resting discharge may rely on only interneuronal sources, while inhibition of TMJ-evoked responses recruits additional input from supraspinal sources. Several sites within this circuit bind E2

and could affect excitability including trigeminal ganglion neurons (TG), GABAergic dorsal horn interneurons, and neurons in the PAG which are critical for RVM outflow.

It is well established that GABA_A receptor-mediated mechanisms are critical for the integration of nociceptive signals by spinal neurons (Yaksh 1989; Sivilotti and Woolf 1994; Knabl et al., 2008). The substrate for GABAergic influence on nociceptive processing by dorsal horn neurons is complex and likely involves presynaptic and postsynaptic mechanisms (Bardoni et al. 2013). Indeed, anatomical (Dumba et al. 1998; DiFiglia and Aronin 1990; Wang et al. 2001) and physiological studies support the notion that GABA acts through presynaptic and postsynaptic mechanisms to alter the excitability of Vc neurons (Wei et al. 2013). Although muscimol decreased the convergent cutaneous RF area of TMJ units in both LE and HE rats, a measured variable generally attributed to central neural mechanisms (Cook et al. 1987), we cannot exclude that BMI acted presynaptically at GABA_A receptors on TMJ afferent terminals as well as postsynaptically on lamina I neurons.

The doses of BMI (0.15 and 1.5 nmol, 30 μ l) applied here were similar to those that caused abrupt motor responses and desynchronized EEG in lightly anesthetized male rats after topical spinal cord application (Zhang et al., 2001), and to intrathecal doses that unmasked tactile hypersensitivity in spinal lamina I units (Seagrove et al., 2004). In the spinal cord preparation in male rats 40 μ M bath application of bicuculline increased dorsal root-evoked polysynaptic EPSCs driven at A fiber strength, while fast monosynaptic EPSCs were not affected suggesting that GABA_A receptor disinhibition most affected interneuronal communication between A β and A δ fiber input and substantia gelatinosa (SG) neurons rather than direct input onto SG neurons (Baba et al., 2003). In the present study the spontaneous activity of TMJ units was increased after high dose BMI ($P < 0.025$) in LE and HE units. Previous reports of increased background firing rates of Vc neurons recorded only from cells in deeper laminae of Vc, examined only effects on cutaneous inputs and used only male rats (Chiang et al., 1999; Takeda et al., 2000). Since we recorded only from TMJ-responsive units, it is possible that some aspects of GABAergic influence on TMJ units are different from units that only receive input from cutaneous sources. The periaqueductal gray-rostral ventromedial medulla (PAG-RVM) is a major endogenous pain control system in the brain and its modulation of nociceptive input displays a significant level of tissue specificity (see Heinricher et al. 2009). The PAG-RVM circuit also is notable for the high density of ER-positive neurons in the PAG (Shughrue et al., 1997; Vanderhorst et al., 2005) and that GABA_A receptor subunits in the PAG vary expression over different stages of the estrous cycle (Lovick 2006). The PAG-RVM system may provide the link to E2-dependent effects of supraspinal control and TMJ-responsive neurons at the Vc/C₁₋₂ region.

Estrogens act through multiple signaling pathways to alter the excitability and connectivity of neurons (McEwen 2001; Scharfman and MacLusky 2006) and a close relationship between estrogen status and GABAergic function is well described for mood and behavior (see Lovick 2006; Backstrom et al., 2013) and reproductive hormone secretion (Wagner et al., 2001). The *gad2* promoter is a target of ER-positive neurons (Hudgens et al., 2009), while in PAG (McCarthy et al. 1995) and hippocampus estrogen status regulates glutamate decarboxylase (GAD) mRNA and protein expression (Nakamura et al., 2004) and dendritic

spine density through a GABA-dependent mechanism (Murphy et al., 1998). A mechanism for E2-mediated disinhibition in hippocampus (Rudick and Woolley 2001; Ledoux and Woolley 2005) and forebrain (Rudick et al., 2003) may involve a decrease in release probability at GABAergic synapses.

The relationship between estrogen status and GABAergic mechanisms at spinal levels is not well defined. A high density of ER-positive neurons is found in superficial laminae, especially within lamina II, at both spinal levels (Papka et al., 2001) and at the Vc/C₁₋₂ region (Amandusson et al., 1996; Pajot et al. 2003; Bereiter et al., 2005). This distribution is similar to that of GABAergic neurons (Todd & McKenzie 1989; Ginestral and Matute, 1993; Polgar and Antal, 1995; Avendano et al., 2005). In caudal Vc nearly 50% of ER-positive neurons also express pre-proenkephalin (Amandusson et al., 1996) or GABA (Bereiter et al., 2007), while in spinal dorsal horn lamina II neurons often co-express enkephalin and GABA (Todd et al., 1992). We found similar levels of GABA_A receptor $\beta 3$ isoform protein at the Vc/C₁₋₂ region in HE and LE rats. However, since these isoforms are found in more than 80% of GABA_A receptors (Benke et al. 1994), it cannot be excluded that changes in other isoforms may have occurred. Recent studies found that E2 treatment caused an upregulation in $\alpha 2$ and $\alpha 6$ subunit expression in the TG and the Vc/C₁₋₂ region (Puri et al., 2011). Alternatively, estrogen status could have affected GABA binding since muscimol binding was reported to be highest in superficial laminae of spinal dorsal horn and increased significantly after exogenous E2 in female rats (McCarthy et al., 1991). Our results were consistent with a reduced GABAergic tone and increased sensitivity to GABA_A receptor activation under HE conditions. However, the mechanism(s) that mediate E2 modulation of GABAergic function that result in altered excitability of superficial laminae neurons is not known. We cannot exclude that responses to GABAergic drugs may have been influenced by estrogenic effects on barbiturate anesthetic levels. However, periodic testing of corneal reflexes throughout the recording session suggested no change in depth of anesthesia. In agreement with our previous studies in male rats (Takeshita et al. 2001), TMJ neurons isolated early versus late in the recording session displayed similar encoding properties. Also, exogenous estradiol had no effect on the clearance rate for pentobarbital in male rats (Knodell et al. 1982).

Pain amplification may result from increased excitatory synaptic drive as well as from reduced inhibition (see Woolf and Salter 2000; Todd 2010). Previously we reported that TMJ-responsive units in superficial laminae of HE rats were markedly inhibited by topical application of the selective NMDA receptor antagonist, AP5, while units from LE rats were not affected (Tashiro et al., 2009a), the opposite effect of GABAergic disinhibition seen in the present study. The gene for the NR2B receptor subunit is a target for the estrogen receptor in rat brain (Watanabe et al., 1999) and NR2B is upregulated in TG neurons that supply the masseter muscle (Dong et al. 2007). Two days after E2 treatment protein levels of the NR1 subunit of the NMDA receptor were increased significantly in lumbar spinal cord and hyperalgesic responses to a visceral stimulus were enhanced (Tang et al., 2008). In behaving mice intrathecal injections of bicuculline caused mechanical hyperalgesia that was blocked by co-administration of AP5, while the spinal dorsal horn protein levels of NR1, phosphorylated NR1 and NR2B subunits were greatly increased (Cao et al., 2011). In the spinal cord preparation GABA_A receptor-mediated disinhibition of superficial laminae

neurons was prevented by bath application of NMDA receptor antagonists (Baba et al., 2003; Torsney and MacDermott, 2006). These studies suggest that GABA_A receptor-mediated disinhibition and glutamatergic pathways are closely linked in nociceptive processing and are significantly influenced by estrogen status. Figure 5 provides a general scheme of how estrogen status may interact with GABAergic mechanisms to modify nociceptive processing in superficial laminae.

In conclusion, these results were consistent with the hypothesis that HE and LE conditions are associated with functional changes in GABAergic tone. Under HE conditions (low GABA tone) local BMI would be expected to have minor effects, while the sensitivity to muscimol would be expected to be high. Conversely, under LE conditions (high GABA tone) the reverse would be expected. The relationship between estrogen status and GABAergic effects on TMJ-responsive neurons is likely complex and may involve different mechanisms for control of resting activity versus evoked responses.

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Highlights

- High E2 enhance TMJ-evoked activity of superficial laminae neurons at Vc/C₁₋₂.
- GABA_A receptor antagonism enhances TMJ-evoked neural activity only under low E2.
- GABA_A receptor activation reduces TMJ-evoked neural activity independent of E2.
- GABA_A receptor protein levels at the Vc/C₁₋₂ are not affected by E2 conditions.
- E2 cause a functional reduction in GABAergic influence on TMJ-responsive neurons.

Figure 1. Peristimulus time histograms displaying examples of the effects of the GABA_A receptor antagonist, BMI, on ATP-evoked responses of TMJ units under (A) HE and (B) LE conditions. Horizontal bars = intra-TMJ injections of ATP (1 mM, 20 μ l); 1 bin = 1 s

Figure 2.

Effects of BMI on total Rmag (A), response duration (B), and response latency (C) of TMJ units recorded in superficial laminae. Sample sizes: HE, $n = 6$; LE, $n = 6$. * $P < 0.05$, ** $P < 0.01$ versus ATP alone; a = $P < 0.05$, b = $P < 0.01$ versus the LE group.

Figure 3. Peristimulus time histograms displaying examples of the effects of the GABA_A receptor agonist, muscimol, on ATP-evoked responses of TMJ units under (A) HE and (B) LE conditions. Horizontal bars = intra-TMJ injections of ATP (1 mM, 20 μl); 1 bin = 1 s.

Figure 4.

Effects of muscimol on total Rmag (A), response duration (B), and response latency (C) of TMJ units recorded in superficial laminae. Sample sizes: HE, $n = 7$; LE, $n = 7$. $**P < 0.01$ versus ATP alone; $a = P < 0.05$, $b = P < 0.01$ versus the LE group.

Figure 5.

Possible circuits and sites for GABA-E2 interactions that could alter the properties of TMJ-responsive neurons in lamina I at the Vc/C₁₋₂ region. Pathways 1a, 1b and 1c represent pre- and post-synaptic actions of GABAergic interneurons in lamina II. Pathway 2 represents descending control pathways from the RVM. Symbols and abbreviations: blue = excitatory interneurons (E); red = inhibitory GABAergic neurons (I); yellow box = ER-positive neurons; lam 1 and lam 2 = laminae I and II of Vc/C₁₋₂ region; P = lamina I projection neurons; PAG = periaqueductal grey region; RVM = rostral ventromedial medulla; TG = trigeminal ganglion.