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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/C_{1-2}) 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 2 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/C_{1-2} 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/C_{1-2} region. Estrogen status may gate the magnitude of GABAergic influence on TMJ neurons at the earliest stages of nociceptive processing at the spinomedullary region.

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AbAbbreviations: aCSF, Artificial Cerebrospinal fluid; ANOVA, analysis of variance; AP5, D-(-)-2-amino-5-phosphonopentanoic acid; BMI, bicuculline methiodide; E2, estradiol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; ER, estrogen receptor; HE, high-dose estradiol; LE, low-dose estradiol; NMDA, *N*-methyl-D-aspartate; NR2B, NMDA receptor subtype; OvX, ovariectomized; PAG, periaqueductal gray; PBS, phosphate-buffered saline; RF, receptive field; Rmag, response magnitude; RVM, rostral ventromedial medulla; SA, spontaneous activity; SD, standard deviation; SG, substantia gelatinosa; TG, trigeminal ganglion; TMJ, temporomandibular joint; TMJD, temporomandibular joint/muscle disorders.

Key words: sex steroids, trigeminal brainstem, nociception, GABAA receptor.

INTRODUCTION

Temporomandibular joint/muscle disorders (TMJD) represent a heterogeneous, often idiopathic, group of pain conditions involving the temporomandibular joint (TMJ) region 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 (LeResche, 1997; Huang et al., 2002; 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 (ERs) affect the susceptibility to develop TMJD (Ribeiro-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/C_{1-2} region (Shigenaga et al., 1986, 1988). The superficial laminae at the Vc/C_{1-2} 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 (OvX) female rats (Tashiro et al., 2007; Okamoto et al., 2013). Although estrogen status markedly affected the response of TMJ-responsive neurons to *N*-methyl-D-aspartate (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

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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). ER-positive neurons also are found in superficial laminae of Vc and colocalize pre-proenkephalin 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 (TG) (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/C_{1-2} neurons under high (HE) and low (LE) estrogen conditions in OvX 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 β3 subunit isoform levels by Western blot. The β3 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 OvX female Sprague–Dawley rats weighing 250–320 g (SLC, Shizuoka, Japan; Sprague–Dawley, Harlan, Indianapolis, IN, USA) 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, USA) dissolved in 200 μl sesame oil for 2 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 CO_2 (3.5–4.5%), mean arterial pressure (MAP, 100–120 mmHg), and body temperature (38 °C) were monitored continuously and maintained within the normal range.

Animals were placed in a stereotaxic frame and dorsal portions of the C_1 and C_2 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 (C_1–C_2) 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 C_2 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 mm^2) or "pinch" stimulus (shorter and stiffer arterial clip, approximately 15 mm^2) 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 2 h after initial anesthesia, TMJ units were recorded from superficial laminae < 200 μm of the dorsal surface within 1.5 mm rostral to the level of the entrance of the C_2 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