Sex-Related Differences in Human Pain and Rat Afferent Discharge Evoked by Injection of Glutamate Into the Masseter Muscle

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1Faculty of Dentistry, The University of Toronto, Toronto, Ontario M5G 1G6, Canada; 2Center for Sensory-Motor Interaction, Laboratory for Experimental Pain Research, Aalborg University, DK-9220 Aalborg; and 3Department of Prosthetic Dentistry and Stomatognathic Physiology, Orofacial Pain Clinic, Royal Dental College, Aarhus University, DK-8000 Aarhus, Denmark

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Cairns, Brian E., James W. Hu, Lars Arendt-Nielsen, Barry J. Sessle, and Peter Svensson. Sex-related differences in human pain and rat afferent discharge evoked by injection of glutamate into the masseter muscle. J Neurophysiol 86: 782–791, 2001. Animal studies have suggested that tissue injury–related increased levels of glutamate may be involved in peripheral nociceptive mechanisms in deep craniofacial tissues. Indeed, injection of glutamate (0.1–1 M, 10 μl) into the temporomandibular region evokes reflex jaw muscle responses through activation of peripheral excitatory amino acid receptors. It has recently been found that this glutamate-evoked reflex muscle activity is significantly greater in female than male rats. However, it is not known whether peripheral administration of glutamate, in the same concentrations that evoke jaw muscle activity in rats, causes pain in humans or activates deep craniofacial nociceptive afferents. Therefore we examined whether injection of glutamate into the masseter muscle induces pain in male and female volunteers and, since masseter afferent recordings were not feasible in humans, whether glutamate excites putative nociceptive afferents supplying the masseter muscle of male and female rats. Injection of glutamate (0.5 M or 1.0 M, 0.2 ml) into the masseter muscle of both men and women caused significantly higher levels of peak pain, duration of pain, and overall pain than injection of isotonic saline (0.2 ml). In addition, glutamate-evoked peak and overall muscle pain in women was significantly greater than in men. In rats of both sexes, glutamate (10 μl, 0.5 M) evoked activity in a subpopulation of masseter muscle afferents (n = 36) that projected to the subnucleus caudalis, an important relay of noxious input from the craniofacial region. The largest responses to glutamate were recorded in muscle afferents with the slowest conduction velocities (2.5–5 m/s). Further, glutamate-evoked masseter muscle afferent activity was significantly greater in female than in male rats. These results indicate that glutamate injection into the masseter muscle evokes pain responses that are greater in women than men and that one possible mechanism for this difference may be a greater sensitivity to glutamate of masseter muscle afferents in females. These sex-related differences in acute experimental masseter muscle pain are particularly interesting given the higher prevalence of many chronic muscle pain conditions in women.

INTRODUCTION

Chronic pain from deep craniofacial tissues is a common affliction, and perhaps as many as 10% of the North American population will suffer, for example, from a temporomandibular disorder (TMD) at some point in their lifetime (Carlsson and LeResche 1995; Drangsholt and LeResche 1999). TMD pain is frequently reflected in masticatory muscle pain, limited jaw motion, and headache, is often poorly localized, and may spread and be referred to the neck, face, or preauricular regions (see Carlson and LeResche 1995; Sessle 2000; Stohler 1995, 1999). There is a much greater prevalence of TMD and related craniofacial pain conditions among women of reproductive age, which suggests that sex-related factors may play a role in the pathogenesis of these conditions. Nonetheless, the mechanisms underlying these sex-related differences in the prevalence of craniofacial pain remain obscure and could involve a variety of factors, including physiological and psychosocial factors (Carlson et al. 1998; Carlsson and LeResche 1995; Dao and LeResche 2000; Dao et al. 1998; LeResche et al. 1997; Maixner et al. 1995; Salonen et al. 1990).

To investigate physiological factors that may underlie the development of pain in deep craniofacial tissues, we have examined the effect of injecting algesic substances into the temporomandibular joint (TMJ) region on the activity of rat jaw muscles. Our most recent evidence indicates that injection of the excitatory amino acid (EAA) glutamate into the TMJ region reflexly evokes activity in the jaw muscles that is greater in female rats than male rats (Cairns et al. 1998, 2001). This result raises the possibility that there may be distinct sex-related differences in some of the mechanisms involved in the processing of sensory inputs from deep craniofacial tissues and that these differences may contribute to the greater prevalence of many chronic muscle pain conditions in women. This result also raises several important questions about peripherally applied glutamate, including whether injection of glutamate in the same concentrations applied to animals would evoke pain in humans and, if so, whether there would be differences between men and women in their reports of pain.

A well-documented experimental model of human masticatory muscle pain has used the injection of hypertonic saline and other algesic chemicals into the masseter muscle to evoke pain responses in healthy subjects (Ernberg et al. 2000; Stohler and...
The present study employed an analogous experimental craniofacial pain model to test the hypotheses that injection of glutamate into the human masseter muscle evokes pain when applied in the same concentrations that evoked jaw muscle responses in the rat and that the intensity of this pain is greater in women than men. In addition, since recording the activity of single masseter muscle afferents was not feasible in humans, muscle afferent recordings were undertaken in rats to characterize the effect of glutamate on slowly conducting, putative nociceptive afferents and to test the hypothesis that glutamate evokes greater activity recorded in muscle afferents of female rats than male rats.

METHODS

Human experiments

SUBJECTS. Healthy volunteers without signs or symptoms of TMD (Dworkin and LeResche 1992) were recruited from university students. The relationship between pain and different concentrations of glutamate following a single injection of glutamate into the masseter muscle was initially determined in 10 women and 8 men. In the next human experiment, repeated injections of glutamate were given into the masseter muscle in 17 women and 16 men. Five men participated in both experiments, which were separated by at least 2 mo. The age of the women (26.0 ± 3.1 yr old, mean ± SE) and men (25.2 ± 2.9 yr old) was not significantly different (P = 0.444, unpaired t-test). The weight of the women and men was 60.8 ± 1.5 kg and 83.2 ± 3.3 kg, respectively (P = 0.001, unpaired t-test). Twelve of the 27 women tested used oral contraceptive medication containing estrogen. The self-reported onset date of the last menses was recorded, and none of the women participating in the study reported irregular menstrual cycles. The study protocol was approved by the local ethics committee in Denmark (Counties of Nordjylland and Viborg) and followed the guidelines set out by the Helsinki Declaration.

GLUTAMATE ADMINISTRATION. Single injection. Sterile solutions of glutamate (0.2 ml; 0.1, 0.5, 1.0 M; pH 6.8) or isotonic saline (0.2 ml; 0.165 M; pH 7.0) were injected over a 10-s period with a 27-gauge hypodermic needle and disposable syringe into the deep masseter muscle midway between its upper and lower border and 1 cm posterior to its anterior border (Svensson et al. 1995). The needle was inserted to a depth until bony contact was made, and then it was retracted about 2 mm before aspiration and injection of the solution. In all experiments, subjects were given standardized instructions and were unaware of which solution was about to be injected (single blind). To avoid sequence effects, injections of glutamate (0.1, 0.5, and 1.0 M) and isotonic saline into the masseter muscles were performed in a randomized fashion during two sessions separated by 1 wk. Numbers were drawn to determine the sequence of substance injection. At each session, the first injection was made into either the left or right masseter muscle, and then 30 min later, a second injection was made into the masseter muscle on the opposite side. The site of the first injection was chosen at random. During the second session, the two remaining substances were injected in association with the same protocol.

Repeated injection. It has been previously demonstrated that a second application of glutamate to the TMJ region of male and female rats 30 min after the initial application could evoke jaw muscle activity of a similar magnitude to that evoked by the initial injection (Cairns et al. 1998, 1999). It was therefore of interest to determine whether the pain following two injections of glutamate (1.0 M) into the masseter muscle would also be of comparable magnitude. These experiments were performed in 17 women and 16 men. The second injection was performed 25 min after the initial glutamate injection in a similar way and at the same site as the first injection.

Pain assessment. The subjects were instructed to continuously rate the pain evoked by the injections of glutamate on an electronic 10-cm visual analog scale (VAS) for 20 min with their jaw at rest. A computer sampled the VAS signals every 5 s. The lower endpoint of the VAS was labeled "no pain at all" and the upper endpoint labeled "the most pain imaginable." Peak pain was measured as the peak VAS score, the area under the VAS curve (VASAUC) was used to obtain a measure of the overall amount of pain, and the onset to offset of pain was determined from the VAS profiles and used as a measure of pain duration. Furthermore, the time-to-peak VAS score was measured in seconds. In addition the subjects were asked to draw the perceived distribution of pain on a map that had a picture of the profile of the face (Fig. 1). These drawings were then digitized (ACECAD, model D9000 + digitizer, Taiwan) to calculate the area of perceived pain in arbitrary units (a.u.) (Svensson et al. 1998).

Rat experiments

ANIMAL PREPARATION. Adult male (n = 16, weight: 362.5 ± 14.0 g) and female (n = 20, weight: 280.5 ± 5.6 g) Sprague-Dawley rats were prepared for acute in vivo recording of trigeminal primary afferent activity under surgical anesthesia (O2: 0.3–0.4 l/min; N2O: 0.6–0.7 l/min; halothane: 1.5–2%) (Cairns et al. 1998, 1999, 2001). A tracheal cannula was inserted and artificial ventilation initiated. The rat’s head was then placed in a stereotaxic frame, the skin over the dorsal surface of the skull was reflected, and a trephination was made on the left side of the skull to allow a microelectrode to be lowered through the brain and into the trigeminal ganglion. A second incision was made and the skin and muscle overlying the brain stem and upper cervical spinal cord were removed, a C1 laminectomy was performed, and the dura overlying the brain stem/cervical spinal cord was removed to facilitate placement of a stimulating electrode in contact with the ipsilateral caudal brain stem (subnucleus caudalis, Vc) or dorsal horn of the upper cervical spinal cord. In female rats, a vaginal lavage was performed, and epidermal cell examination revealed that...
five were in estrus, nine in metestrus, and six in diestrus (Frye et al. 1992; Martinez-Gomez et al. 1994).

After completion of all surgical procedures, the halothane level was slowly reduced (1–1.3%) until noxious pressure applied to the hind paw could induce a weak flexion reflex of the hindlimb to ensure that an adequate level of anesthesia was maintained for the duration of the experiment. Heart rate and body core temperature were continuously monitored throughout the whole experiment and kept within the physiological range of 330–430/min and 37–37.5°C, respectively. All surgeries and procedures were approved by the University of Toronto Animal Care Committee in accordance with the regulations of the Ontario Animal Research Act (Canada).

STIMULATION AND RECORDING TECHNIQUES. Single trigeminal primary afferent unit activity within the trigeminal ganglion was recorded by a parylene-coated tungsten microelectrode (2 MΩ, A-M Systems, Carlsborg, WA). The microelectrode was slowly lowered into the brain under stereotaxic control (3.5–4 mm anterior to the interaural line, 3–4 mm lateral to the midline) until unit discharge was observed in response to light brush stimuli applied to the craniofacial region. Trigeminal primary afferent units were usually found 7–8 mm below the surface of the cerebral cortex. A blunt probe was applied as a mechanical search stimulus over the masseter muscle while the electrode was slowly lowered in an attempt to identify trigeminal primary afferents with muscle mechanoreceptive fields. When a unit was found that appeared to respond to mechanical stimulation of the masseter muscle, the skin overlying the mechanoreceptive field was pulled gently away from contact with the muscle, and brush, pinch, and pressure stimuli were applied directly to the skin surface. If the unit did not respond to any of these cutaneous stimuli, then the mechanoreceptive field was considered to lie within the muscle. It was observed that subsequent insertion of the catheter needle used to inject glutamate into the masseter muscle evoked a spike discharge in all muscle afferents identified in this manner (Paintal 1960). At the completion of some experiments, the skin overlying the masseter muscle was surgically excised, and it was confirmed that mechanical and electrical stimuli applied directly to the muscle tissue could also evoke activity in the afferent that previously had been characterized by mechanical and glutamate stimulation in the experiment.

Previous anatomical studies indicated that small-diameter masseter muscle afferents project to the ipsilateral Vc and dorsal horn of the cervical spinal cord (Arvidsson and Raappana 1989; Capra and Wad 1989; Nishimori et al. 1986; Shigenaga et al. 1988). To confirm that masseter muscle afferents in the current study projected to this region, constant-current electrical stimuli (50-μs biphasic pulse, range 10–80 μA, 0.5 Hz) were applied to a stimulating electrode (2 MΩ, parylene-coated tungsten electrode, A-M Systems) lowered into the ipsilateral caudal brain stem (1–1.5 mm lateral to the midline, 0–5 mm caudal to the obex, depth 0–0.5 mm below the brain stem surface). The stimulating electrode was moved medially (0.1-mm steps) and rostrocaudally (0.5-mm steps) in the caudal brain stem until electrical stimulation evoked an antidromic action potential with an invariant latency (<0.2-ms variability) and the ability to follow high-frequency electrical stimuli (≥100 Hz) (Cairns et al. 1996; Dostrovsky et al. 1981). The initial electrical stimuli were applied 5 mm caudal to the obex. If stimulation at this location did not evoke an antidromic action potential, the stimulating electrode was moved rostrally toward the obex until either an antidromic action potential was evoked or electrical stimuli had been applied unsuccessfully up to the level of the obex. Antidromic action potentials were collided with orthodromic action potentials evoked by mechanical stimulation of the masseter muscle, to confirm the projection of the masseter muscle afferent to the caudal brain stem. At the end of the experiment, the distance between the stimulating and recording electrodes was measured and divided by the latency of the antidromically evoked response of an afferent to give an estimation of conduction velocity (CV) of the recorded afferent.

After the above characterization of each afferent was completed, the needle tip of a catheter (a 27-gauge needle connected by polyethylene tubing to a Hamilton syringe, 50 μL) was carefully inserted into the masseter muscle. This catheter was used to inject glutamate (0.5 M, 10 μl, adjusted to pH 7.4 with NaOH; Sigma, St. Louis, MO). In all rat experiments, baseline primary afferent activity was recorded for 10 min prior to injection of any substance into the mechanoreceptive field. Glutamate was then slowly injected into the mechanoreceptive field (over a 5-s period), and any resulting primary afferent activity was monitored for 30 min after the injection.

As in the human experiments, the effects of repeated injections into the masseter muscle were also studied. In six rats, a second injection of glutamate (0.5 M) into the masseter muscle was made 30 min after the first. In an additional six rats, the same concentration of the amino acid GABA (0.5 M, 10 μl, adjusted to pH 7.0 with NaOH; Sigma) was injected 30 min after the initial injection of glutamate. The use of GABA also served to assess the possible effect of osmosality since GABA and glutamate have comparable molecular weights (103 and 146, respectively).

TERMINAL PROCEDURES. At the end of each experiment, rats were killed with the agent T61 (Hoechst, Canada). Electrical lesions were made in the brain stem of some rats by applying a monopolar, monophasic current pulse of 20 μA for 20 s. The brain stem was then removed, and thin sections of the brain stem and upper cervical spinal cord (100 μm) were cut with a vibratome and viewed under a microscope.

DATA ANALYSIS. The activity of identified primary afferents was amplified (Afferent gain: ×1,000; bandwidth 30–1,000 Hz) and fed into a computer equipped with a CED 1401 Plus board and analysis software (Spike 2; Cambridge Electronic Design, Cambridge, UK). Recorded primary afferent activity was stored electronically and analyzed off-line.

Peristimulus time histograms (PSTHs; 1-min bins) were constructed from the recorded primary afferent discharge. Mean baseline afferent discharge was calculated by averaging the 1st 10 bins prior to injection of glutamate. Mean baseline afferent activity was subtracted from each bin of the PSTH to yield residual afferent discharge. The area under the glutamate-evoked afferent response curve (AUC) was calculated by the summation of the residual afferent discharge after glutamate injection (Cairns et al. 1998, 1999).

Statistics

Values are reported as means ± SE in the text and figures. In the human experiments the VAS scores were analyzed with a two-way ANOVA (gender and repeated injections or different glutamate concentrations as factors). The ANOVA was followed by post hoc comparisons with the use of Tukey tests. Weight and age was compared between women and men with the use of unpaired t-tests.

In the rat experiments, an unpaired t-test was used to compare the mean weight and conduction velocity of the male and female rats. A Mann-Whitney rank sum test was used to compare the peak and AUC values for male and female rats, and a Kruskal-Wallis one-way ANOVA on ranks was used to compare the AUC values for female rats in different estrus stages, since AUC and peak data were not normally distributed. A Wilcoxon signed-rank test was used to compare the masseter muscle afferent responses when repeated injections were made.

A Pearson product moment correlation was performed to assess the relationship between weight and VAS scores or afferent responses within male and female subject groups. In all tests, the level of significance was set at P < 0.05.
RESULTS

Human experiments

GENERAL FEATURES. All subjects described a deep, aching type of pain after the injections of 1 M glutamate. The majority of both the men and women did not localize the pain solely to the injected masseter muscle but also reported that it spread to involve other craniofacial tissues such as the TMJ and teeth ipsilateral to the site of injection (Fig. 1). There was a significant difference in the perceived area of pain ($F = 4.935, P = 0.033$, 2-way ANOVA) with significantly greater areas being measured on the drawings of the women ($P = 0.034, Tukey test$). There was, however, no significant difference area of pain produced by the first and second injections of glutamate ($F = 0.205, P = 0.654$, 2-way ANOVA) and no significant interaction between gender and repeated injection.

There were no significant differences in glutamate-evoked pain between the women taking oral contraceptive medication ($n = 12$) and those who did not ($n = 15$, $P = 0.789$, unpaired $t$-test). The women were not tested in any specific phase of the menstrual cycle, and the overall test dates were evenly distributed throughout the menstrual cycle. Although the mean weight in the men was significantly greater than that in the women ($P = 0.001$, unpaired $t$-test), there was no significant correlation between weight and glutamate-evoked pain in the men ($r_{men} = -0.208, P = 0.407$, Pearson correlation), but there was a significant correlation between weight and pain evoked by glutamate in the women ($r_{women} = 0.443, P = 0.03$, Pearson correlation).

SINGLE INJECTION. Different concentrations of glutamate were tested in 8 men and 10 women. The peak pain ratings and the $V_{AS}_{AUC}$ showed a significant difference between the men and women ($F = 14.812, P = 0.001$, 2-way ANOVA) with significantly higher pain responses for the women than for men ($P = 0.001$, Tukey test; Fig. 2, A and B). There was no significant gender difference for the time to peak pain ($F = 0.004, P = 0.948$, 2-way ANOVA). Furthermore, there were no significant interactions between sex and concentration, although the peak pain ratings and $V_{AS}_{AUC}$ in the women, but not the men, were slightly higher following injections of 1.0 M glutamate compared with 0.5 M glutamate.

The different concentrations of glutamate evoked significantly different pain responses in the group of 8 men and 10 women (Fig. 2, A–C). The peak pain rating, the $V_{AS}_{AUC}$, and the duration of pain were all influenced by the concentration ($F = 13.825, P = 0.001$, 2-way ANOVA). There was no significant interaction between gender and different concentrations of glutamate. Isotonic saline (308 mOsm/l) caused no or very low levels of pain when injected into the masseter muscle of the men or women (Fig. 2, A–C). Compared with isotonic saline and 0.1 M (186 mOsm/l) glutamate, injection of 0.5 M (950 mOsm/l) and 1.0 M (2148 mOsm/l) glutamate into the masseter muscle caused significantly higher levels of peak pain, duration of pain, and overall ($V_{AS}_{AUC}$) pain in both men and women ($P < 0.001$, Tukey test; Fig. 2, A–C). There was also a significant difference between the different concentrations of glutamate and isotonic saline for the time-of-peak VAS ($F = 10.508, P = 0.003$, 2-way ANOVA). The time-for-peak VAS for both the 0.5 M glutamate (50.6 ± 7.1 s, mean ± SE) and 1.0 M glutamate (44.4 ± 7.9 s) was significantly later than the time-for-peak VAS for the isotonic saline (14.7 ± 4.2 s) and 0.1 M glutamate (20.8 ± 6.0 s; $P < 0.011$, Tukey tests).

There was no significant difference between the 0.5- and 1.0-M concentrations of glutamate for any of the measured VAS parameters ($P > 0.933$, Tukey test).

REPEATED INJECTION. The pain responses evoked by two injections of 1.0 M glutamate into the masseter muscle were investigated in 16 men and 17 women. For all glutamate-evoked pain responses, there were significant differences between the women and men (Fig. 3, A–C). The peak VAS was significantly higher ($F = 10.508, P = 0.003$, 2-way ANOVA), the $V_{AS}_{AUC}$ significantly higher ($F = 14.6, P = 0.001$, 2-way ANOVA), and duration of response significantly longer ($F = 9.486, P = 0.004$, 2-way ANOVA) in the women than men. There was no significant sex-related difference in the time-to-peak VAS ($F = 0.409, P = 0.527$, 2-way ANOVA). There was no significant interaction between sex and repeated injections for any of the VAS parameters.

The peak pain ratings were significantly increased from the first to the second injection ($F = 7.254, P = 0.011$, 2-way ANOVA; Fig. 3A). No effects of repeated injections were
observed for the VAS AUC (F = 0.481, P = 0.493, 2-way ANOVA), duration of pain (F = 0.447, P = 0.509, 2-way ANOVA; Fig. 3, B and C) or time-to-peak VAS (F = 2.607, P = 0.117).

**Rat experiments**

**GENERAL FEATURES.** A total of 42 trigeminal primary afferents that responded to the blunt probe applied over the masseter muscle were recorded from the trigeminal ganglion of adult rats of either sex (Fig. 4). None of these afferents responded to brush, pinch, and pressure stimuli applied directly to the skin surface overlying the masseter muscle. All 42 masseter muscle afferents had antidromically identified projections to the caudal brain stem. Based on the distance from the obex where the electrical stimuli were applied, in concert with histological reconstruction of selected stimulation sites, all masseter muscle afferents could be antidromically activated from either the Vc or dorsal horn of the upper cervical spinal cord (Fig. 5A).

Prior to injection of glutamate, 13 masseter muscle afferents exhibited some ongoing spike discharge (mean: 5.2 ± 1.9 spikes/min). Injection of glutamate (0.5 M, 10 µl) into the masseter muscle evoked activity in 36 of the 42 afferents. All masseter muscle afferents, both glutamate-sensitive (mean CV: 10.7 ± 1.0 m/s) and glutamate-insensitive (mean CV: 9.6 ± 1.3 m/s) had CVs in the Aδ range (Fig. 5C). There was an inverse relationship between the CV and the AUC (Fig. 5C) such that the largest responses to glutamate were recorded in muscle afferents with the slowest CVs (2.5–5 m/s).

**SINGLE INJECTION.** A total of 16 and 20 glutamate-sensitive muscle afferents were recorded from male and female rats, respectively. Glutamate injection into the masseter muscle induced afferent activity with a latency of 3–10 s and a duration of 10–1,800 s. The glutamate-evoked afferent discharge exhibited a burst-pause firing pattern in 65 and 50% of masseter muscle afferents recorded in female and male rats, respectively. The proportion of afferents recorded in male and female rats with an action potential discharge greater than two SDs above their preinjection baseline activity (~95% confi-
sex-related difference in glutamate-evoked muscle pain

FIG. 5. General properties of the 42 masseter muscle afferents examined in the present study. A: all afferents had conduction velocities (CVs) in the Aδ afferent range (2.5–35 m/s). B: the majority of afferents had projections that terminated between 2 and 3 mm caudal to the obex. C: the scatter plot illustrates the relationship between conduction velocity and magnitude of afferent response. The magnitude of glutamate-evoked afferent discharge was inversely related to CV.

There was no significant difference in the CV for afferents recorded in male (10.8 ± 1.4 m/s) and female (10.8 ± 1.3 m/s) rats (P = 0.961, unpaired t-test). However, the peak response was significantly greater in female (328 ± 109 spikes/min) than in male rats (42 ± 21 spikes/min, P = 0.003, rank sum test; Fig. 7). The AUC value calculated for glutamate-evoked afferent responses in female rats (494 ± 173 spikes × min) was also significantly greater than that in male rats (43 ± 14 spikes × min, P = 0.003, rank sum test). There were no significant differences in AUC values calculated for female rats in diestrus (n = 6, 751 ± 253 spikes × min), estrus (n = 5, 407 ± 246 spikes × min), and metestrus (n = 9, 393 ± 131 spikes × min; H = 4.883, P = 0.087, 1-way ANOVA on ranks). Glutamate-evoked masseter afferent response levels were not significantly correlated with body size in male (r_{male} = -0.25, P = 0.361, Pearson correlation) or female rats (r_{female} = -0.30, P = 0.204, Pearson correlation).

REPEATED INJECTION. In a subgroup of six masseter afferents (5 female, 1 male; CV: 12.1 ± 2.3 m/s), a second injection of glutamate was made 30 min after the initial injection of glutamate to compare the activity evoked by repeated injections (Fig. 8A). The magnitude of activity evoked by the second injection of glutamate (mean AUC_2: 626 ± 379 spikes × min) was not significantly different from that evoked by the initial injection of glutamate to the masseter muscle (mean AUC_1: 826 ± 474 spikes × min; P = 0.219, Wilcoxon signed-rank test). To investigate whether injection of a different amino acid would be equally effective in evoking masseter muscle afferent activity, six masseter afferents (3 female, 3 male; CV: 11.3 ± 2.4 m/s), GABA (0.5 M; 750 mOsm/l) was injected into the masseter muscle 30 min after the initial injection of glutamate (Fig. 8B). The use of GABA also served to assess the possible effect of osmolarity since GABA and glutamate solutions were of comparable osmotic strength. Injection of GABA (mean AUC_2: 5 ± 2 spikes × min) evoked significantly less activity than the initial injection of glutamate (mean AUC_1: 220 ± 180 spikes × min; P = 0.031, Wilcoxon signed-rank test) in this subgroup of afferents.

FIG. 6. The scatter plot illustrates the percent of afferents with activity >2 SD units above baseline (~95% confidence interval) in male (○) and female (●) rats. Time 0 indicates the injection of glutamate. The percentage of active afferents for the 1st 5 min after glutamate injection was higher in female than male rats. In some afferents in both males and females, activity was enhanced, relative to baseline, for more than 25 min after injection of glutamate.
bar graph (binwidth 1 min) summarizes the overall mean activity (the PSTH illustrates the activity of a single afferent (CV: 2.6 m/s), and the calibration bar:

**DISCUSSION**

**Glutamate-evoked responses in humans and rats**

The present study shows that injection of glutamate into the masseter muscle can evoke pain in humans and also demonstrates that there are sex-related differences in both a human and animal model of deep craniofacial pain. We interpret these findings to be due, at least in part, to a local effect of glutamate, since in the human experiments, pain was reported in the craniofacial region on the same side as the injection, and in the animal experiments glutamate injections activated masseter muscle afferents.

**Possible mechanisms**

Several mechanisms may have been responsible for the glutamate-evoked masseter muscle pain. To avoid the confound of low pH, glutamate solutions were adjusted to pH 6.8–7.4 prior to their injection, thus it is unlikely that the pain experienced was related to the pH of the solution (Steen et al. 1992). The role of mechanical distention in pain responses to glutamate was assessed by the injection of the same volume of isotonic saline that resulted in very low pain scores. Unlike pH, it was not possible to adjust the osmolarity of the glutamate solutions, and it has been shown that application of hypertonic saline to the human masseter muscle results in pain reports that are qualitatively similar to those recorded in the present study after intramuscular injection of high concentrations of glutamate (Stohler and Kowalski 1999; Svensson et al. 1995, 1996, 1998; Zhang et al. 1993). Thus it is possible that the osmotic strength of the glutamate solutions played a role in the reported pain responses. However, the injection of GABA of comparable osmolarity and concentration to glutamate evoked significantly less afferent activity than glutamate, suggesting that elevated osmotic strength is not likely to be a major factor responsible for the excitant effect of peripherally applied glutamate.

Activation of peripheral EAA receptors appears to play a role in mechanisms of craniofacial pain transduction in rats and might also have contributed to the pain reported in the present study by human volunteers when the EAA glutamate was injected into the masseter muscle (Cairns et al. 1998; Yu et al. 1996). A subpopulation of slowly conducting (Aδ and C) muscle afferents in both humans and animals can be activated by locally applied intense pressure and algesic chemicals and are thought to be associated with muscle nociception (Fock and Mense 1976; Kaufman et al. 1982; Kumazawa and Mizumura 1977; Marchettini et al. 1996; Mense 1977, 1993; Paintal 1960; Simone et al. 1994). We found that injection of glutamate into the rat masseter muscle evoked activity in Aδ mechanoreceptive afferents that were shown to project to the caudal brain stem, a region documented to be a critical relay of nociceptive input from jaw muscles as well as other craniofacial tissues (Capra and Wax 1989; Nishimori et al. 1986; Shigenaga et al. 1988; see reviews by Hu et al. 1997; Sessle 2000). Glutamate has been shown to excite trigeminal afferents through activation of N-methyl-D-aspartate (NMDA) and non-NMDA receptors (MacIver and Tanelian 1993; Pelkey and Marshall 1998; Puil and Spiegelman 1988). Further, reflex muscle responses can be evoked by activation of either NMDA or non-NMDA receptors in deep craniofacial tissues (Cairns et al. 1998). This evidence suggests that activation of peripheral EAA receptors may excite slowly conducting masseter muscle nociceptors that contribute to pain responses in humans and is consistent with the association between the development of hyperalgesia and elevated tissue levels of glutamate elsewhere in the body (Carlton et al. 1995; Jackson et al. 1995; Lawand et al. 1997, 2000; McNearney et al. 2000).

In our halothane-anesthetized rats, all masseter muscle afferents that responded to both mechanical stimulation of the muscle and glutamate injection had conduction velocities in the Aδ range. The inability to identify C afferents appears unrelated to the anesthetic employed, since halothane has been reported to increase the excitability of trigeminal C afferents but decrease the excitability of trigeminal Aδ afferents (MacIver and Tanelian 1990). It may be that masseter muscle C afferents were not activated by the mechanical search stimuli employed. Nevertheless, it remains to be determined whether C afferents innervating the masseter muscle would be similarly activated by glutamate.

**Effect of repeated injection**

The present findings indicate that the area under the VAS pain curve in humans and afferent activity in rats evoked by

FIG. 8. A comparison of the afferent activity evoked by injection of glutamate or GABA into the masseter muscle. In A, the PSTH (binwidth 1 s) illustrates the activity of a single afferent (CV: 4.2 m/s), and the bar graph (binwidth 1 min) summarizes the overall mean activity (n = 6), evoked by 2 injections of glutamate ( acquainted with 1, 0.5 M, 30-min interval) into the masseter muscle. In B, the PSTH illustrates the activity of a single afferent (CV: 2.6 m/s), and the bar graph (binwidth 1 min) summarizes the overall mean activity (n = 6), evoked by injection of glutamate ( acquainted with 1) followed, 30 min later by GABA ( acquainted with 0.5 M). Note that afferent activity evoked by a 2nd injection of glutamate was similar to that evoked by the 1st injection of glutamate. In contrast, injection of GABA evoked significantly less afferent activity than glutamate. Vertical calibration bar: A, 400 spikes; B, 200 spikes; error bars: SE.
Sex-related differences in glutamate-evoked responses

Women rated injection of glutamate (0.5 and 1.0 M) into the masseter muscle significantly more painful than men. Body weight and the cross-sectional area of the masseter muscle is greater in men than women, and it is conceivable that the greater VAS scores reported by women could have been due to their smaller size (Close et al. 1995). However, we found no correlation between weight and pain response in the men, and the correlation analysis in women showed that larger weights were actually associated with higher pain scores. The clinical significance of the latter finding is not known at present. Furthermore, afferent discharges in male and female rats were not correlated with body size, which suggests that muscle size as such unlikely played a significant role in the observed differences in pain ratings or muscle afferent activity.

Neither men nor women appeared to be able to discriminate between masseter muscle injection of 0.5 M and 1.0 M glutamate. However, the higher VAS scores in women than men were mirrored by a greater glutamate-evoked muscle afferent discharge in female rats than male rats. It appears therefore that sex-related differences in response to glutamate may be at least partly explained by the greater responses of masseter muscle primary afferents in females to glutamate. Nevertheless, noxious stimulation of the deep tissues can also induce prolonged changes in the excitability of both spinal cord and trigeminal brain stem nociceptive neurons that may be greater in female rats (see Bereiter et al. 1998; Hoheisel and Mense 1989; Hu et al. 1992; Sessle 1999, 2000). The larger areas of perceived pain reported by women after injection of glutamate into the masseter muscle also suggest that central integrative mechanisms may contribute to the observed sex-related differences. The size of perceived pain areas is proportional to pain intensity (Graven-Nielsen et al. 1997), and thus the greater pain intensity reported by women may have been responsible for their reports of larger areas of perceived pain. However, sex-related differences in focused attention were not investigated in the present study, and this factor could also have played a role in determining the size of perceived pain areas.

Previous studies have found that women report greater pain after noxious thermal, electrical, or mechanical cutaneous stimuli than do men, although because the reported magnitude of these differences has often been small, the significance of this difference has been questioned (Berkley 1995; Dao and LeResche 2000; Fillingim and Ness 2000a; Riley et al. 1998). Nevertheless, while men and women have a similar ability to discriminate the intensity of noxious cutaneous thermal stimuli, women report lower pain thresholds and greater pain during repetitive thermal stimuli (Edwards et al. 1999; Fillingim et al. 1998). The greater temporal summation of noxious cutaneous thermal stimuli in women has been interpreted as evidence of a sex-related difference in central integration (Fillingim et al. 1998; Maixner et al. 1995).

Fluctuations in pain sensitivity during the menstrual cycle are difficult to detect, particularly with small numbers of subjects (Fillingim and Ness 2000a,b). In the present study, we tested a relatively small number of subjects, and test dates were evenly distributed throughout the menstrual cycle of women who were not taking oral contraceptives. The pain responses of these women were not significantly different from the pain responses of women who reported taking estrogen-containing contraceptives. In addition, no estrous cycle stage-related differences in glutamate-evoked afferent activity were noted in female rats. Thus we are not in a position to draw any conclusions about the influence of menstrual cycle stage on magnitude of glutamate-evoked masseter muscle pain in women. Nevertheless, we do not exclude the possibility that menstrual cycle stage could exert some influence on the magnitude of glutamate-evoked masseter muscle pain; an effect that might be revealed by examining a larger population of women (Dao et al. 1998; LeResche et al. 1997).

Clinical relevance

The prevalence of chronic pain conditions that include symptoms of masseter muscle pain, such as TMDs and fibromyalgia syndrome, is significantly greater in women than men (Bennett 1995; Carlton et al. 1998; Carlsson and LeResche 1995; Dao and LeResche 2000; LeResche et al. 1997; Maixner et al. 1995). Interestingly, while mechanical pain pressure thresholds for the masseter muscle are lower in fibromyalgia and TMD sufferers than in age- and sex-matched controls, sex-related differences in mechanical pain pressure threshold have not been consistently found, and several methodological differences may account for this (see Bush et al. 1993; Dao and LeResche 2000; Isselee et al. 1998; Plesh et al. 1998). In this regard, our finding that injection of glutamate into the masseter muscle evokes greater pain responses in women than men suggests that noxious chemical as opposed to mechanical stimuli may be more useful for examining sex-related differences related to the development of myalgia. In addition, the injection of noxious chemicals in humans allows the recording of both a temporal and a spatial pain profile (Figs. 1 and 2).

As noted above, peripheral glutamate receptors have been implicated in the activation of primary afferents and in the development of hyperalgesia after tissue injury (Cairns et al. 1998; Carlton et al. 1995; Du et al. 2001; Jackson et al. 1995; Lawand et al. 1997, 2000; MacIver and Tanelian 1993; McNearney et al. 2000; Pelkey and Marshall 1998; Puil and Spigelman 1988; Yu et al. 1996). In concert with the present study, such findings point to a potential role for peripheral EAA receptors in the modulation of pain arising from deep craniofacial tissues such as the masseter muscle and TMJ. Further, our finding of a sex-related difference in pain responses to peripherally injected glutamate suggests that if selective peripheral EAA receptor antagonists can be developed, they could be used to determine whether peripheral EAA receptor activation is involved in the development or mainte-
nance of masseter muscle pain related to TMD or fibromyalgia syndrome in women.

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