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SEX-RELATED DIFFERENCES IN NMDA-EVOKED RAT MASSETER MUSCLE AFFERENT DISCHARGE RESULT FROM ESTROGEN-MEDIATED MODULATION OF PERIPHERAL NMDA RECEPTOR ACTIVITY

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Abstract

In the present study, the hypothesis that sex-related differences in glutamate-evoked rat masseter muscle afferent discharge may result from estrogen-related modulation of peripheral NMDA receptor activity and/or expression was tested by examining afferent fiber discharge in response to masseter injection of NMDA and the expression of NR2A/B subunits by masseter ganglion neurons in male and female rats. The results showed that injection of NMDA into the masseter muscle evoked discharges in putative mechanonociceptive afferent fibers and increased blood pressure that was concentration-dependent, however, a systemic action of NMDA appeared responsible for increased blood pressure. NMDA-evoked afferent discharge was significantly greater in female than in male rats, was positively correlated with plasma estrogen levels in females and was significantly greater in ovariectomized female rats treated with a high dose (5 µg/day) compared to a low dose (0.5 µg/day) of estrogen. Pre-treatment of high dose estrogen-treated-ovariectomized female rats with the Src tyrosine kinase inhibitor PP2 did not affect NMDA-evoked afferent discharge. NMDA-evoked afferent discharge was attenuated by the antagonists ketamine and ifenprodil, which is selective for NR2B containing NMDA receptors. Fewer masseter ganglion neurons expressed the NR2A (16%) subunit as compared with the NR2B subunit (38%), which was expressed at higher frequencies in intact female (46%) and high dose estrogen-treated ovariectomized female (60%) rats than in male (31%) rats. Taken together, these results suggest that sex-related differences in NMDA-evoked masseter afferent discharge are due, at least in part, to an estrogen-mediated increase in expression of peripheral NMDA receptors by masseter ganglion neurons in female rats.

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Keywords

Nociception; Craniofacial; Estrogen; Temporomandibular; Sex; Trigeminal

INTRODUCTION

Temporomandibular disorders (TMD) are a group of clinical conditions which have as their chief complaint craniofacial pain. While a majority of clinical TMD cases involve pain in the masticatory muscles, in most patients the underlying pathophysiological basis for this pain is unclear (Lobbezoo-Scholte et al., 1995, Sessle, 1999, Svensson and Graven-Nielsen, 2001, Stohler, 2002, Yap et al., 2003). Further complicating the clinical picture of TMD is the greater prevalence of this condition amongst women than men (Dao and LeResche, 2000, Phillips et al., 2001, Nekora-Azak, 2004). This sex-related difference in TMD prevalence suggests that sex-hormones may play a role in the development of TMD, and indeed a link between the female sex hormone estrogen and TMD-related pain has been proposed (LeResche et al., 1997, 2003, Nekora-Azak, 2004, Sherman et al., 2005). It remains to be determined, however, whether there is an interaction between estrogen and muscle pain mechanisms.

One mechanism that could contribute to muscle pain in TMD is elevated tissue levels of the excitatory amino acid glutamate. Glutamate levels have been reported to be elevated in tendons and muscles in association with sports injuries or exercise-evoked muscle pain (Alfredson and Lorentzon, 2002, Tegeder et al., 2002, Rosendal et al., 2004), however, whether the level of glutamate changes in masticatory muscles during TMD has yet to be determined. The nociceptive effect of elevated tissue glutamate levels in tendons, joints and the viscera appears to be mediated by activation of peripheral ionotropic and/or metabotropic glutamate receptors (Lawand et al., 1997, Cairns et al., 1998, Alfredson et al., 2001, McRoberts et al., 2001, Zhang et al., 2002). Taking advantage of these findings, we have developed human and animal models of TMD-like muscle pain that employ injection of glutamate into masticatory muscles as a non-inflammatory noxious stimulus (Cairns et al., 2001, Svensson et al., 2003). Glutamate injection into the masseter muscle has been found to evoke greater pain in women than in men and greater afferent discharge in female rats than in male rats, which suggests that a peripheral mechanism may underlie sex-related differences in glutamate-evoked pain (Cairns et al., 2001, 2002, Svensson et al., 2003). Activation of peripheral NMDA receptors by glutamate is responsible, at least in part, for both pain responses in humans and afferent discharge in rats (Cairns et al., 2003). Although the peripheral receptor mechanisms which underlie sex-related differences in response to exogenous application of glutamate to deep craniofacial tissues remain to be elucidated, estrogen can significantly enhance NMDA receptor-mediated neuronal responses in the central nervous system through an effect on the NR2B subunit (Woolley et al., 1997, Foy et al., 1999). This finding suggests that sex-related modulation of peripheral NMDA receptor activity might play an important role in the propensity for elevated tissue levels of glutamate to evoke greater pain responses in females. Therefore, in the present study the hypothesis that sex-related differences in glutamate-evoked afferent discharge result from estrogen-related modulation of the activity and/or expression of peripheral NMDA receptors in female rats was tested.

EXPERIMENTAL PROCEDURES

Intact

Adult Sprague-Dawley rats of either sex (weight: 225–385 g) were prepared for acute in vivo recording of trigeminal afferent discharges as has been previously reported (Cairns et al., 2001, 2002, 2003). A tracheal cannula was inserted and artificial ventilation initiated with a rodent ventilator. Gas anesthesia (O_2 : 0.3–0.4 l/min; isoflurane 2.0–2.5%) was administered

through a tracheal cannula. The femoral or carotid artery was cannulated for monitoring blood pressure and, in certain rats, the femoral vein was also cannulated to permit intravenous administration of drugs. The rat's head was placed in a stereotaxic frame, the skin over the dorsal surface of the skull reflected and a trephination made on the right side of the skull to allow a microelectrode to be lowered through the brain and into the trigeminal ganglion. The skin, muscle and dura overlying the brainstem and upper cervical spinal cord were removed to permit placement of a stimulating electrode in contact with the brainstem for antidromic activation of masseter muscle afferent fibers projecting to the caudal brainstem. Heart rate, mean blood pressure, expired CO₂ and body core temperature were continuously monitored throughout the whole experiment and kept within the physiological range of 300–400/min, 60–80 mmHg, 20–50 mmHg and 36.8–37.1 °C, respectively. All procedures were performed in adherence with the principles of the Canadian Council on Animal Care and were approved by the University of British Columbia Animal Care Committee.

Gonadectomized female rats

Ovariectomized female Sprague-Dawley rats were purchased from Charles River (Saint-Constant, Quebec, Canada). Each rat received an estrogen pellet (Innovative Research of America, Sarasota, FL) capable of producing a constant dose of either 0.5 (low dose, n = 5) or 5.0 µg/day (high dose, n = 11) over a 21-day period. Pellets were inserted subcutaneously at the back of the neck. Ten days after pellet injection, rats were prepared for acute in vivo afferent fiber recordings or histological examination as described.

In the hippocampus, high levels of estrogen act through the Src tyrosine kinase pathway to enhance NMDA receptor function and long-term potentiation (Woolley et al., 1997, Foy et al., 1999, Bi et al., 2000, 2001). To investigate whether estrogen might alter peripheral NMDA receptor activity by activation of the Src tyrosine kinase pathway, ovariectomized female rats treated with high dose estrogen were also given a systemic dose of the Src tyrosine kinase inhibitor PP2 (0.5 mg/kg) at least 2 hours prior to examining the response of masseter afferent fibers to NMDA injection into the masseter muscle. This dose of PP2 has been demonstrated to inhibit the action of Src tyrosine kinases in vivo without affecting baseline systemic blood pressure (Beaucage et al., 2005).

Stimulation and Recording Techniques

Single trigeminal afferent unit activity within the trigeminal ganglion was recorded by a parylene-coated tungsten microelectrode (2 MΩ, A-M Systems Inc., Carlsborg, WA, USA). A blunt probe was applied as a mechanical search stimulus to the skin over the masseter muscle while the electrode was slowly lowered in an attempt to identify trigeminal afferent fibers with a masticatory muscle mechanoreceptive field. 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 receptive field was considered to lie within the muscle. Semmes-Weinstein monofilaments (Stoelting, Wood Dale, IL, USA) were used to estimate the mechanical activation threshold of afferent fibers.

To confirm that all masticatory muscle afferent fibers in the current study projected to the caudal brainstem and thus were putative mechanonociceptive afferent fibers, constant-current electrical stimuli (100 µsec biphasic pulse, range 10–90 µA, 0.5 Hz) were applied to a stimulating electrode (2 MΩ, parylene-coated tungsten electrode, A-M Systems Inc., Carlsborg, USA) that had been lowered into the ipsilateral caudal brainstem to evoke antidromic action potentials (Cairns et al., 2001, 2002, Svensson et al., 2003). Antidromic action potentials were collided with orthodromic action potentials evoked by mechanical stimulation of the masseter

muscle to verify the projection of the masticatory muscle afferent fiber to the caudal brainstem. 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 fiber to give an estimation of the conduction velocity (CV) of the recorded afferent fiber.

Solutions were injected intramuscularly into the mechano-receptive field of identified masseter muscle afferent fibers via a catheter composed of a 26-gauge needle connected by polyethylene tubing to a Hamilton syringe. Single 10 μ l intramuscular injections of NMDA (5, 50, 160, 500 or 1600 mM) were made in order to assess the concentration-afferent response relationship for male and female rats. To confirm that the high concentrations of NMDA injected were exciting afferent fibers through a peripheral NMDA receptor mechanism, the effect of the NMDA receptor antagonist ketamine on NMDA-evoked afferent discharge in male and female rats was also investigated. Repeated injections of drug solutions (10 μ l) were made into the masseter muscle at 30 min intervals. The initial injection contained NMDA alone, while the subsequent injection contained NMDA alone or in combination with ketamine (1, 5, 10 or 20 mM). We have previously demonstrated that co-injected ketamine (up to 10 mM) does not exert non-specific antagonist actions on masseter afferent fibers, e.g. sodium channel blockade (Cairns et al., 2003). The effect of ifenprodil (100 μ M), which is a non-competitive NMDA NR2B subunit selective antagonist (Williams, 1993, Avenet et al., 1996) on NMDA-evoked afferent discharge was also assessed in a similar manner. All solutions were adjusted to ~ pH 7.4 prior to injection. Chemicals were obtained from Sigma (St. Louis, MO, USA).

Baseline primary afferent fiber activity was recorded for 10 min prior to each injection into masseter muscle. Intramuscular injections were then made slowly into the muscle (over a 5 s period) and any resulting afferent activity monitored for 10 min after the injection. When possible, multiple afferent fiber experiments were undertaken in each individual rat to minimize the number of animals required for experimentation.

To investigate whether changes in blood pressure induced by injection of NMDA into the masseter muscle were due to a local rather than a systemic effect, 4 rats (2 male, 2 female) were injected with an amount of NMDA (5 μ mol diluted in 0.15 ml saline) equivalent to the dose (~ 2.5 mg/kg) provided by masseter muscle injection of 10 μ l of 500mM NMDA.

Measurement of Plasma Estrogen

The plasma concentration of the unconjugated form of 17 β -estradiol was measured with a commercially available radioimmunoassay kit (ICN Biomedical, Inc., Costa Mesa, CA, USA).

Immunohistochemistry

Fast blue (2 %, 10 μ l) was injected bilaterally into the masseter muscle of isoflurane-anesthetized rats (3 male, 3 female, 3 5.0 μ g/day estrogen-treated ovariectomized female rats). Seven days after injection, rats were euthanized with an overdose of pentobarbital (100 mg/kg) and then perfused with 120 ml cold saline followed by 120 ml of paraformaldehyde (4%). The right and left trigeminal ganglia were removed and cut into sections (40 μ m) with a vibratome.

A purified mouse monoclonal antibody specific for the NR2A (n = 3 male, 3 female and 3 high dose estrogen treated ovariectomized female ganglia) or NR2B (n = 3 male, 3 female and 3 high dose estrogen treated ovariectomized female ganglia) subunits was purchased from BD Signal Transduction (San Diego, CA). Sections were treated with 5% normal goat serum (NGS) in phosphate buffered saline for 1 h followed by overnight incubation with either mouse monoclonal antibody against NR2A (1:300) or NR2B subunit in phosphate buffered saline containing 1% normal goat serum. The next morning sections were washed several times with

phosphate-buffered saline and then incubated for 1 h at room temperature in the dark in the presence of CY3-conjugated goat anti-mouse IgG (diluted 1:400) for antibody localization. After several washes in buffer, all sections were mounted on slides with cover slips and were visualized with a Leica DM fluorescent microscope. The specificity of receptor-like immunoreactivity was confirmed by omission of the monoclonal mouse (NR2A or NR2B) antibody.

Data Analysis

The activity of identified afferent fibers was amplified (gain: 1000x; 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, U.K.). Cumulative afferent discharges were calculated by subtracting the summed action potential discharges for 10 min after injection from the baseline (spontaneous) afferent discharges 10 min prior to injection. The cumulative afferent discharges evoked by the second injection were divided by the cumulative afferent discharges evoked by the first injection to yield relative cumulative activity.

Peak change in blood pressure after injection of NMDA into the masseter muscles was calculated by subtracting the maximum blood pressure obtained during the pre-injection baseline period from the maximum blood pressure obtained after injection. The duration of increased blood pressure was calculated by the time period where the blood pressure was greater than two times above the standard deviation of the mean baseline.

Statistics

The data collected were not normally distributed, and thus, non-parametric statistical tests were employed. For multiple comparisons, a Kruskal-Wallis ANOVA on ranks with a post-hoc Dunn's test when appropriate was used to assess statistical significance. For comparisons between males and females, the Mann-Whitney test was employed. Spearman correlation on ranks was performed to assess the relationship between estradiol levels and mechanical threshold, afferent CV, as well as NMDA-evoked cumulative afferent discharges. Data in the text and tables is reported as the median and interquartile range (square brackets).

RESULTS

Baseline Response Characteristics

The response properties of 227 putative mechanonociceptive masseter afferent fibers were assessed in intact, adult male and female rats (Table 1). There was no significant sex-related difference in the CV or mechanical threshold of these afferent fibers. No significant correlation between afferent mechanical threshold and plasma estrogen was found for either sex.

NMDA-Evoked Effects

Injection of NMDA into the masseter muscle evoked activity in masseter afferent fibers in a concentration-dependent manner (Figure 1). When data from all afferent fibers were examined, the cumulative afferent discharge was significantly greater in female rats than in male rats at 160 mM (Figure 1). If only slowly conducting afferent fibers (CV < 10 m/s) were included in the analysis, NMDA-evoked cumulative afferent discharge was significantly greater in female rats for both 160 mM and 1600 mM.

Injection of NMDA into the masseter muscle also increased blood pressure in a concentration-dependent manner (Figures 2). There were, however, no significant sex-related differences in NMDA-evoked increases in blood pressure. Intravenous injection of NMDA (5 μ mol) evoked similar increases in blood pressure (median peak change: 20.6 [17.3–22.0] mmHg; median duration: 1214 [633–1552] seconds) to those evoked by intramuscular injection of 500mM

NMDA, which suggests that the pressor effect of intramuscularly injected NMDA was due to a systemic effect.

NMDA Receptor Antagonists

To confirm that the high concentrations of NMDA used in the present study were acting on the peripheral NMDA receptor, the non-competitive NMDA receptor antagonist ketamine was co-injected with NMDA in female and male rats. Control experiments indicated that a second injection of 500 mM NMDA alone into the masseter muscle 30 min after the first injection re-excited the afferent fiber, although the magnitude of afferent discharge after a second injection was often decreased (Figure 3A). Ketamine caused a concentration-related suppression of NMDA-evoked afferent fiber discharge in both sexes (Figure 3B).

Additional experiments were undertaken to determine the sensitivity of NMDA-evoked afferent discharge to blockade by the NR2B subunit containing NMDA receptor antagonist ifenprodil in an additional 8 female and 8 male rats (Williams, 1993, Avenet et al., 1996). Compared with injection of NMDA alone, co-injection of ifenprodil significantly attenuated NMDA-evoked masseter afferent fiber discharge in both sexes (Figure 3B).

Influence of Plasma Estrogen Levels

The influence of estrogen levels on the NMDA concentration-response curve for slowly conducting afferent fibers recorded in male and female rats is illustrated in Figure 4. By excluding slowly conducting afferent fibers recorded in female rats with estrogen concentrations of less than 60 pg/ml (the approximate median level of estrogen in male and female rats), the concentration-response curve of the slowly conducting afferent fibers shifted to the left, which suggests an increased response of slowly conducting afferent fibers to NMDA injection with increasing estrogen level in females. In contrast, excluding slowly conducting afferent fibers recorded in male rats with estrogen concentrations less than 60 pg/ml tended to shift the concentration-response curve to the right, which suggests a decreased afferent fiber response to NMDA injection with increasing estrogen level in males.

The relationship between masseter afferent discharge evoked by the first injection of 500 mM NMDA from the ketamine experiments and plasma estrogen concentration was also examined in both male and female rats. In female rats, there was a significant positive correlation ($r = 0.312$, $P = 0.012$) between plasma estrogen concentration and NMDA-evoked afferent discharge ($n = 52$ afferent fibers; estrogen concentration: 72 [53–117] pg/ml). In contrast, in male rats, there was no significant correlation between plasma estrogen and NMDA-evoked masseter discharge ($n = 52$ afferent fibers; estrogen concentration: 49 [38–67] pg/ml).

To confirm the apparent influence of estrogen levels on NMDA-evoked afferent activity, additional experiments were undertaken where ovariectomized female rats were treated with a high dose (5 $\mu\text{g/day}$) or a low dose (0.5 $\mu\text{g/day}$) of estrogen for 10 days prior to examining the response of masseter afferent fibers to NMDA injection into the masseter muscle. The median estrogen concentration produced by high dose replacement therapy (282 [167–403] pg/ml, $n=9$) was significantly greater than that produced by low dose replacement therapy (132 [87–147] pg/ml, $n=5$). There was no significant difference in the CV or mechanical threshold of afferent fibers between rats treated with the high dose ($n = 5$ rats) and the low dose ($n = 5$ rats). NMDA-evoked afferent discharge, however, was significantly greater after high dose replacement therapy when compared with low dose replacement therapy (Figure 5). Further, NMDA-evoked afferent discharge was significantly correlated with estrogen levels in rats treated with low dose estrogen ($r = 0.74$, $P = 0.013$) but not in rats treated with high dose estrogen.

To investigate whether the effect estrogen might be mediated through phosphorylation of the peripheral NMDA receptor by Src tyrosine kinases, ovariectomized female rats were treated with a high dose (5 µg/day, n = 4 rats) of estrogen for 10 days and then given a systemic dose of the Src tyrosine kinase inhibitor PP2 (0.5 mg/kg) two hours prior to examining the response of masseter afferent fibers to NMDA injection into the masseter muscle. There was no effect of PP2 pretreatment on the magnitude of NMDA-evoked afferent discharge (Figure 4). However, administration of PP2 did reduce the median NMDA-induced peak change in blood pressure by almost 40% (7.9 [6.0–12.7] mmHg), which indicates that the PP2 did exert a systemic effect.

NR2 Receptor Subunit Expression

The NR2B subunit of the NMDA receptor has been implicated in estrogen modulation of NMDA receptor function in central nervous system neurons (Woolley et al., 1997, Foy et al., 1999, Bi et al., 2000, 2001). To examine which NR2 receptor subunits are expressed by trigeminal ganglion neurons that innervate the masseter muscle, fast blue was injected into the masseter muscle and fast blue positive trigeminal ganglion neurons were examined for expression of the NR2A or NR2B subunits. Post-mortem examination of the injection sites revealed that all of the fast blue dye remained within the masseter muscle. Examination of sections of trigeminal ganglion after fast blue was injected into the masseter muscle of intact male and female rats yielded a total of 645 positive neurons that had estimated diameters of 3 µm to 70 µm. The population distribution of estimated cell diameters for these masseter afferent neurons was similar to previously reported findings (Figure 6A) (Ambalavanar et al., 2003, 2005, Takeda et al., 2005). In 6 ganglia (3 male, 3 female), expression of the NR2A subunit was examined and it was found that only 16% of masseter afferent neurons (n = 41 of 252 cells) expressed this subunit. The frequency of NR2A positive fast blue labeled masseter afferent neurons in female rats (12%) was lower than in male rats (20%). In 6 ganglia (3 male, 3 female) expression of the NR2B subunit was examined and it was found that 38% of masseter afferent neurons (n = 139 of 392 cells) exhibited cell membrane expression of this subunit (Figure 6). The frequency of NR2B positive fast blue labeled masseter afferent neurons in female rats (46%) was greater than in male rats (31%).

To investigate whether NR2A or NR2B expression is influenced by estrogen, 3 ganglia from ovariectomized female rats treated with the high dose (5 µg/day) estrogen for 10 days were examined. It was found that 18% (n = 30 of 163 cells) and 60% (n = 132 of 219 cells) of masseter afferent neurons exhibited cell membrane expression of the NR2A and NR2B subunits, respectively.

DISCUSSION

The present study provides novel data demonstrating that injection of NMDA into the masseter muscle evokes discharge in putative mechanonociceptive afferent fibers and increases blood pressure. Together with previous results that demonstrate injection of glutamate into the human masseter muscle causes pain in part through activation of peripheral NMDA receptors, these findings indicate that activation of peripheral NMDA receptors is a noxious stimulus which excites slowly conducting afferent fibers in masticatory muscles (Cairns et al., 2003, 2006, Dong et al., 2006). Injection of the endogenous NMDA receptor agonist glutamate into the masseter muscle evokes both activity in slowly conducting afferent fibers in rats and pain in human subjects which is significantly greater in females than in males (Cairns et al., 2001, 2002, Svensson et al., 2003). However, since glutamate can activate both peripheral NMDA and non-NMDA ionotropic receptors in deep craniofacial tissues, either or both receptor subtypes might mediate the observed sex-related differences in response to glutamate injection into the masseter muscle (Cairns et al., 1998, 2002). The results of the present study demonstrate

that there is a significant sex-related difference in the response of masseter muscle afferent fibers to activation of peripheral NMDA receptors that is strongly correlated with plasma estrogen levels. One mechanism underlying this sex-related difference in response to peripheral NMDA receptor activation appears to result from an estrogen-mediated increase in the expression of peripheral NMDA receptors by masseter ganglion neurons.

In the central nervous system there is good evidence for a significant interaction between NMDA receptor activity and estrogen. The magnitude of NMDA receptor-mediated long term potentiation (LTP) in the rat hippocampus is increased with both exogenous and natural cyclic elevation of estrogen levels (Woolley et al., 1997, Foy et al., 1999, Bi et al., 2000, 2001). Both a genomic effect of 17 β -estradiol, which involves increased expression of NMDA receptors and a non-genomic mechanism in which 17 β -estradiol activates Src tyrosine kinases to phosphorylate tyrosine residues on the NR2 subunit of the NMDA receptor, have been implicated (Woolley et al., 1997, Bi et al., 2000, 2001, Cyr et al., 2001). Increased NR2 subunit phosphorylation is also observed both when ovariectomized females are treated with a high dose of estrogen and in intact, normally cycling female rats during proestrus (the stage when estrogen levels are highest) when compared to diestrus (when estrogen levels are lower) (Bi et al., 2001). Phosphorylation of the NR2 subunit increases NMDA receptor open time and probability of opening without an effect on single channel conductance, resulting in increased NMDA-mediated currents (Wang et al., 1996, Salter and Kalia, 2004).

The present study indicates that estrogen levels in normally cycling rats were positively correlated with NMDA-evoked masseter afferent discharge and that treatment of ovariectomized female rats with high dose estrogen could significantly increase NMDA-evoked masseter afferent discharge. Medium-sized trigeminal ganglion neurons express estrogen receptors (ER α) in a sex- and estrous cycle-dependent manner (Ambalavanar et al., 2003, Bereiter et al., 2005, Puri et al., 2005), which suggests that the co-expression of estrogen and NMDA receptors necessary for an interaction likely exists in at least some masseter ganglion neurons. Our findings of an increased frequency of expression of NR2B subunit of the NMDA receptor in masseter ganglion neurons from female rats compared with male rats, and the further increase of NR2B expression in gonadectomized female rats treated with high dose estrogen, suggest that genomic mechanisms are at least partly responsible for the observed sex-related differences in NMDA-evoked masseter afferent discharge. In contrast, systemic administration of the Src tyrosine kinase inhibitor PP2 did not affect NMDA-evoked afferent discharge in estrogen-treated ovariectomized female rats, which suggests that if a non-genomic mechanism that involves estrogen-mediated phosphorylation of peripheral NMDA receptors is contributing to the observed sex-related differences in NMDA-evoked afferent discharge, it does not appear to be mediated through activation of this particular pathway.

Consistent with previous studies that have identified both NR1 and NR2B subunits of the NMDA receptor in a variety of tissues (colon, skeletal muscle and tendon), on dorsal root ganglion neurons and on the unmyelinated terminal endings of cutaneous and visceral nerve fibers (Sato et al., 1993, Carlton et al., 1995, 2003, Alfredson et al., 2001, Marvizon et al., 2002, Du et al., 2003, Lai and Ip, 2003, Cahusac et al., 2005), we found that masseter ganglion neurons expressed the NR2B subunit. Further, NMDA-evoked afferent discharge was significantly attenuated by the NR2B selective antagonist ifenprodil, which implies that this subunit is a component of functional peripheral NMDA receptors in putative mechanonociceptive afferent fibers that innervate the masseter muscle. It is thought that peripheral NMDA receptors play a role in mechanical pain transduction mechanisms since activation of these receptors leads to mechanical sensitization of visceral and skeletal muscle afferent fibers as well as to nocifensive behavior in rats and pain in humans (McRoberts et al., 2001, Beirith et al., 2002, Cairns et al., 2002, 2006, Ro, 2003, Svensson et al., 2005). However, peripheral NMDA receptors likely also play a role in the transduction of innocuous mechanical

stimuli by both cutaneous and deep tissue afferent fibers (McRoberts et al., 2001, Chaban et al., 2004, Cahusac et al., 2005). In the craniofacial region, trigeminal mesencephalic nucleus neurons which innervate spindle afferent fibers of masticatory muscle express NR2A and NR2B subunits (Pelkey and Marshall, 1998, Turman et al., 1999) and activation of peripheral NMDA receptors has been suggested to contribute to innocuous mechanical activation of slowly adapting type I mechanosensory whisker afferent fibers (Cahusac et al., 2005). In deep somatic tissues, dorsal root ganglion neurons which innervate the viscera express primarily the NR2B subunit and the mechanical sensitivity of these visceral afferent fibers can be modulated by NMDA receptor antagonists (McRoberts et al., 2001, Marvizon et al., 2002, Chaban et al., 2004). Taken together, these findings suggest that peripheral NMDA receptors modulate the response of afferent fibers to both innocuous and noxious mechanical stimulation and thus may be important in the development and maintenance of certain forms of acute and chronic pain, particularly those which emanate from deeper tissues.

Pressor responses have been advocated as a reliable correlate of pain in awake, freely moving and lightly anesthetized rats, but have been reported to be gradually attenuated and disappear at a depth of anesthesia similar to that employed in the present study (Taylor et al., 1995). Although it was found that injection of NMDA into the masseter muscle of deeply anesthetized rats resulted in a significant and reproducible pressor response, subsequent investigation indicates that this pressor response can be replicated by intravenous injection of NMDA. Although few studies have reported on the cardiovascular effects of systemically administered NMDA, it appears that systemic (intravenous) administration of low doses of NMDA (< 1 mg/kg) decreases blood pressure, while administration of higher doses (~ 12 mg/kg) can cause transient (< 60 seconds) increases in blood pressure (Sitniewska et al., 2003, Kaczynska and Szereda-Przestaszewska, 2004). In comparison, we saw more prolonged increases in blood pressure after administration of ~ 2.5 mg/kg NMDA, although the underlying mechanisms for this phenomenon were not investigated in this study and thus remain to be determined.

There is interest in developing analgesics that target peripheral NMDA receptors and may prove effective in the treatment of certain types of nociceptive and neuropathic pain (Warncke et al., 1997, Carlton et al., 2003, Sawynok, 2003). The results of this study indicate that concentrations of ≥ 10 mM ketamine can almost entirely attenuate peripheral NMDA receptor-evoked afferent discharge in both sexes in rats, however, recent findings suggest that this concentration of ketamine may attenuate glutamate evoked-masseter muscle pain better in men than in women (Castrillon et al unpublished data; Cairns et al., 2003, 2006). Ifenprodil, which is selective for NR2B subunit containing NMDA receptors over NR2A subunit containing NMDA receptors (Avenet et al., 1996, Williams, 1993), significantly decreased NMDA-evoked afferent discharge in both sexes. Taken together, these findings suggest that the utility of peripherally administered ifenprodil in the treatment of muscle pain in conditions such as TMD merits further investigation.

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Figure 1.

The graph illustrates the NMDA concentration-response relationship for afferent discharge. Slowly conducting afferent fibers (slow fibers) were masseter afferent fibers with CVs less than 10 m/s. Note that NMDA-evoked cumulative masseter afferent activity was greater in females (n=47 rats) than in males (n=53 rats). Each dot represents a population median (5–12 responses). Lines indicate the interquartile range. * $P < 0.05$ Mann-Whitney rank sum test comparing males and females

Figure 2.

A. The line plots illustrate examples of the effect of injection of 5 μ mol NMDA into the masseter muscle and intravenously via the femoral vein on the blood pressure. Note that injection of NMDA by either route resulted in a similar increase in blood pressure. **B.** The graph illustrates the concentration-response relationship for blood pressure evoked by injection of NMDA into the masseter muscle. Blood pressure responses increased in a dose-related manner in both sexes. Although there was a tendency for NMDA-induced increases in blood pressure to be of shorter duration in female rats than in male rats, this difference was not statistically significant.

Figure 3.

The line plots and peristimulus histograms in **A.** show an example of the effect of repeated injection of NMDA (500 mM, $\blacktriangle$) into the masseter muscle on a slow A δ masseter afferent fiber (CV = 5.4 m/s). The inset illustrates a collision (*) between the orthodromic action potential generated by pressing the masseter muscle and the antidromic action potential generated by electrical stimulation of the caudal brainstem for this masseter afferent fiber. Note that afferent discharges were evoked with repeated injections of NMDA. The line and scatter plot in **B.** illustrates the effect of adding increasing concentrations of ketamine to the second injection of NMDA on relative cumulative activity. Co-injection of ketamine significantly suppressed NMDA-evoked afferent discharge in a concentration-related manner that did not

exhibit sex-related differences. The bar graph in **C**, compares the relative cumulative activity induced by repeated injection of NMDA alone (C; n = 10 female, (white bar) and 10 male (black bar) rats) to that evoked by co-injecting ifenprodil with NMDA (I; n = 8 female (white bar) and 8 male (black bar) rats) for the second injection. Note that ifenprodil significantly reduced NMDA-evoked afferent discharge in both sexes. Each bar represents a median. Lines indicate interquartile range. #: $P < 0.05$, Kruskal Wallis ANOVA on ranks, Dunns test for combined male (n=50 rats) and female (n=47 rats) data. *: $P < 0.05$ Mann-Whitney rank sum test comparing males and females.

Figure 4.

The graph shows the effect of eliminating afferent fibers recorded in animals with low estrogen (< 60 pg/ml) on the NMDA concentration-response curve in males (n=53 rats) and females (n=47 rats). For clarity, only the median NMDA-evoked discharge is shown.

Figure 5.

The bar graphs illustrate the median 500 mM NMDA-evoked afferent discharge in intact females (on left, n=47 rats) and in ovariectomized females (on right, n=14 rats) treated with estrogen. NMDA-evoked afferent discharge was significantly greater in females with estrogen levels above 120 pg/ml when compared with females with estrogen levels under 60 pg/ml. NMDA-evoked afferent discharge was significantly greater in ovariectomized female rats treated with a high dose of estrogen (E5), than in those treated with a low dose of estrogen (E0.5), however, there was no effect of pre-treatment with the Src tyrosine kinase inhibitor PP2 (PP2) on NMDA-evoked afferent discharge in ovariectomized female rats treated with a high dose of estrogen. #, $P < 0.05$, Kruskal Wallis ANOVA on ranks, Dunns test comparing intact females rats; *, $P < 0.05$, Kruskal Wallis ANOVA on ranks, Dunns test comparing estrogen treated ovariectomized rats

Figure 6.

A. The bar graph illustrates the frequency versus estimated cell diameter for all trigeminal ganglion neurons labeled with fast blue in intact male and female rats. **B.** An example of a cluster of masseter ganglion neurons positive for NR2B-like immunoreactivity (a), fast blue (b) and both labels (c) from the intact female rat is shown. The asterisk indicates one cell nucleus and the arrows indicate NR2B-like immunoreactivity on the cell membrane and the scale bar indicates 50 μm . **C.** The histogram illustrates the frequency of NR2B labeling versus estimated cell diameter in masseter afferent ganglion neurons. Note that the frequency of labeling was higher in neurons from intact females ($n = 3$ ganglia) than from intact males ($n = 3$ ganglia).

Table 1

Population characteristics of masseter afferent fibers are presented. Numbers are medians with interquartile range indicated in the square brackets.

Sex	Weight (g)	Estrogen (pg/ml)	Mechanical Activation Threshold (g)
Female (n=83)	260 [250–272]	80.1 [56.5–117.6]	26 [10–60] (n=110)
Male (n=89)	*318 [280–339]	*55.3 [40.4–72.2]	26 [15–100] (n=117)

* P < 0.05 Mann-Whitney rank sum test