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Sexual dimorphism of Murine Masticatory Muscle Function

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Abstract

(1) Objective—To determine if gender distinctions of force generating capacity existed in murine masticatory muscles.

(2) Design—In order to investigate the effect of sex on force generating capacity in this muscle group, an isolated muscle preparation was developed utilizing the murine anterior deep masseter. Age-matched male and female mice were utilized to assess function, muscle fiber type and size in this muscle.

(3) Results—Maximum isometric force production was not different between age-matched male and female mice. However, the rate of force generation and relaxation was slower in female masseter muscles. Assessment of fiber type distribution by immunohistochemistry revealed a threefold decrease in the proportion of myosin heavy chain 2b positive fibers in female masseters, which correlated with the differences in contraction kinetics.

(4) Conclusions—These results provide evidence that masticatory muscle strength in mice is not affected by sex, but there are significant distinctions in kinetics associated with force production between males and females.

Keywords

Temporomandibular; masseter; fiber type; myosin heavy chain; gender

INTRODUCTION

Craniofacial muscles are distinct from those in the limbs and trunk in both their developmental origin and their protein/molecular composition. Muscles of the craniofacial region arise from the head prechordal mesoderm or neural crest, whereas limb and trunk muscle originate in the paraxial mesoderm flanking the neural tube (1). In addition, human masticatory muscles present a unique fiber type and size distribution compared to limb and trunk muscles (2-4), a feature which has also been documented in many other species, including mice, rabbits, and pigs (5-7).

The process of mastication involves several different muscles to open and close the jaw as well as laterally position the teeth. In humans, the masseter, temporalis and medial pterygoid muscles are the primary jaw closers, which develop the most force and are the site of pain after chewing. In rodents, it is the masseter that is the major force-producing jaw-closing muscle,

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which has been demonstrated by electromyographic monitoring of all muscles of mastication in rats (8). The masseter muscle fiber orientation is not uniform and can be parallel or perpendicular to the temporomandibular joint (TMJ). Therefore, in situ examination of function must account for multiple fiber direction and the types of movements generated by contraction, or must examine the force of jaw closure as an indirect method of muscle contractile force.

Several *in vivo* models have been utilized to assess functional changes in mastication and have provided insight into the myogenic component of muscle disorders such as those involving the TMJ (9,10). An *ex vivo* model enables study of contractile characteristics of the muscle independent of changes in neural input or tissue environment, and may aid in determining the role of the contractile apparatus in the onset of TMD.

The goal of this investigation was to determine if the murine masticatory muscle function differs by sex. To date, this has been examined at the single fiber level, but not in an isolated whole muscle. Therefore, we developed an isolated anterior deep masseter (ADM) preparation to determine if force generation differs between male and female mice.

MATERIALS & METHODS

All procedures were approved by the University of Pennsylvania IACUC. Adult C57Bl/6mice (N=8 per sex, age 6 months), a widely accepted strain for muscle testing, were utilized for this study. Mice were sacrificed by carbon dioxide asphyxiation. The superficial masseter tendon was cut from the mandible, and the muscle was pulled back and removed to reveal the deep masseteric region. The mandible and the zygomatic process of the maxilla were cut at the sites shown in Figure 1, maintaining the deep masseter between these bones. The entire bone-muscle unit was separated from the rest of the skull and placed in oxygenated (95% O₂ and 5% CO₂) Ringer's solution, maintained at pH 7.4 and 22°C. Each sample was trimmed under a dissecting microscope along the direction of the fibers to a width of approximately 2 mm, revealing an origin at the rostral maxillary portion of the zygomatic arch and an insertion along the masseteric ridge of the mandible. Silk sutures were threaded over the four bony corners of the sample, forming superior and inferior "trapezes" for suspension. Figure 1 illustrates the site of the dissected muscle and the preparation of the ADM for mechanical measurements.

The ADM muscle preparation was attached by the sutures to a fixed hook and a force transducer (Model 300C; Aurora Scientific, Ontario, CAN) within a temperature controlled bath of oxygenated Ringers, pH 7.4, 22°C as previously described (11). The suspended muscle was held between two platinum electrodes (Aurora Scientific, Ontario, CAN). Supramaximal, single pulse stimulations determined the optimal muscle length (L_o). Muscle length was determined between the bony insertions at the center of the muscle preparation using calipers. The ADM then received a single pulse followed by a train of 120 Hz-800msec pulses 5 seconds later to measure maximum isometric twitch (Pt) and tetanic (Po) force, respectively. Initial experiments determined that this frequency was sufficient to generation fused tetanic stimulation in both male and female muscle preparations (data not shown). The stimulation pattern was delivered 3 times with 5 minute rests between each measurement. The muscles were then placed in a fresh bath of oxygenated Ringer's solution containing 0.1% procion orange (Reactive Orange 14; Sigma-Aldrich, St Louis, MO) for 20 minutes to enable visualization of fibers at each edge of the muscle preparation that had been damaged during the dissection(12). This is a membrane impermeant dye that can only enter fibers when the sarcolemma is disrupted. Muscle samples were washed in Ringers without procion orange, then dissected free of the bones. All tissue samples were blotted, weighed, pinned at resting length and the orientation of the fibers were aligned along the length of the mounting block. mounting Samples were embedded in mounting medium (Tissuetek; Sakura, Torrance, CA), and then rapidly frozen in melting isopentane and stored at -80°C for morphological analysis.

Absolute cross sectional area was determined using the muscle mass, Lo, and the density of muscle (1.06 g/mm^3) ($\text{mass}/(\text{Lo} \times \text{muscle density})$), and used to calculate maximum specific force ($\text{Force}/\text{cross sectional area}$, Sp Force). The calculated cross-sectional area was confirmed with image analysis (see below). Force generation analysis was performed using software associated with the mechanics apparatus (DMA Version 3.2; Aurora Scientific, Aurora, Ontario, CAN).

Frozen muscle cross sections ($10\mu\text{m}$) were utilized to assess damaged fibers, distribution of fiber area, and fiber type. Orientation of the cross section was confirmed during sectioning under a light microscope. Image acquisition and analysis was performed on an epifluorescence microscope (Leica DMR, Bannockburn, IL) and associated software (OpenLab; Improvision, UK). For each muscle, an image of the entire cross section was acquired. Slides utilized for analysis of fiber damage were washed in phosphate buffered saline (PBS), air-dried, then covered with mounting media containing 4'-6-Diamidino-2-phenylindole (DAPI) to identify nuclei (Vectashield, Burlingame, CA). Damaged fibers were ones that had allowed procion orange entry. The proportion of the muscle at the mid-belly that had damaged fibers was calculated and subtracted from the absolute cross sectional area to determine the functional cross sectional area. Muscle preparations with more than 15% damage were eliminated from the study. Fiber area analysis was achieved using immunohistochemistry and an antibody recognizing laminin as previously described (13). The imaging software provided further confirmation that the fibers were not cut obliquely during sectioning. Identification of 2b, 2a, and I fibers was achieved utilizing monoclonal antibodies recognizing the relevant myosin heavy chains (MHC) (14). These were BF-F3 for MHC 2b, SC-71 for MHC 2a, and BA-F8 for MHC I. Approximately 2000 fibers were analyzed per muscle sample for fiber area and fiber type.

Statistical analysis for muscle mechanics utilized unpaired t tests for comparisons between male and female muscles. Fiber type and area analysis utilized a Mann Whitney test for nonparametric comparisons due to the lack of normally distributed data. Statistical significance was accepted for $P < 0.05$.

RESULTS

There was no significant difference between sexes for the cross-sectional area and mass of the ADM muscle preparation (Table I). This is due to the fact that the dissection of the muscles was the primary determinant of the width of the preparation. However, the muscle length of female ADM muscles was shorter than those isolated from male mice ($P=0.001$). The edges of the ADM preparations contained fibers inevitably damaged by dissection, even though care was taken to cut the muscle in the direction of fiber orientation. Damaged fibers were visible by procion orange fluorescence in the muscle cross sections. Sex did not affect the proportion of damaged fibers. Analysis of fiber damage for combined male and female preparations revealed that $11.8 \pm 1.0\%$ of the fibers were positive for procion orange uptake (Figure 2), and did not contribute to force generation.

Examples of single twitch and fused tetanic stimulation from a male and female ADM muscle is shown in Figure 3. Single twitch force (Pt) was not significantly different between sexes, but tended to be lower and slower in the female ADM muscles (Table 1). This resulted in a significant decrease in the twitch:tetanus ratio in female muscles compared to male muscles ($P=0.019$) (Table 1). The sex distinction was more apparent with tetanic force (Figure 3B), where the rate of force generation (max dP/dt , $P=0.009$) and relaxation after contraction ($1/2 \text{ Rel}$, $P=0.014$) were significantly slower and longer in female muscles (Table 1). While the kinetic parameters differed between the sexes, there was no apparent difference in the amount

of maximum force generated (Po) or the force per cross sectional area (Sp Force) with respect to sex (Table 1).

The distribution of fiber area and proportion of fiber type was measured to determine if there was a correlation to sex or the mechanical measurements. Fiber cross sectional areas did not have Gaussian distributions, and so analysis of the median fiber size was utilized for comparisons between males and females. For all fibers, median fiber area was significantly larger in the female muscle preparations (Figure 4A). Fibers expressing either MHC I, 2a, or 2b were analyzed individually. MHC I positive fibers were rare in both male and female ADM muscles (< 1% total fibers; 5-10 fibers per cross section) and were found in and surrounding the muscle spindle, and within intrafusal muscle fibers as previously observed in several mammals (15). They were not analyzed further because there were too few to make statistical comparisons of fiber area or proportion. Regional differences of fiber type distribution were evident, where MHC 2b fibers were more prevalent on the lateral surface of the muscle, and MHC 2a fibers were found throughout the cross sections. There was no apparent difference along the rostral-caudal axis. The median cross sectional area of both the MHC 2a and MHC 2b fiber population were larger in females than in males ($P<0.001$), although male muscles contained the maximum MHC 2b fiber area (Figure 4A). The largest difference between sexes was the threefold decrease in the proportion of MHC 2b fibers in the ADM muscles isolated from female mice, leading to significantly more MHC 2b fibers in males (Figure 4B). Other fiber types were not assessed, due to the unavailability of specific antibodies.

DISCUSSION

This study developed an isolated murine ADM muscle preparation to address if masticatory muscle function differed with respect to sex. We found the isometric specific force of these muscles was not affected by sex. However, the contractile properties were faster in males than females, particularly in rise and relaxation with tetanic stimulation. This is most likely caused by the increased proportion of MHC 2b fibers in the male ADM muscles, which is the fiber type that produces the fastest shortening velocity (16-18). This observation is consistent with previous characterizations of fiber type distributions in murine masticatory muscles (5,19), where male muscles are comprised of a greater proportion of MHC 2b fibers both in number and proportional area. We have now extended the work from previous studies to evaluate function of male and female murine masseters.

Several animal models for masticatory muscles have been evaluated for their relevance to human tissues (6). The biomechanics and development of mastication has been studied in hamsters, rabbits, and pigs, among others, which aid in understanding the normal and pathological processes involved in human mastication (20-22). Larger species may be advantageous in the study of masticatory movements because of the scale and geometrical similarity of the craniofacial skeleton to humans. However, there is a distinct advantage to utilizing small rodents for these studies, thus enabling the combination of functional assays with transgenic mouse models. Further, because all mammals possess the same developmental lineage for craniofacial muscles, molecular findings are likely to translate across species. The isolated ADM has been utilized before to examine rat masticatory muscle function (23,24), and the same techniques were adopted for our investigation in the mouse. As a more molecular understanding of pathologies associated with the craniofacial complex develops, transgenic manipulation with mice will become key to determining the genetic influence on muscle function.

The size of the ex vivo muscle preparation is important to optimize. First, if the dissected portion of the ADM is too large, then the central region becomes anoxic when the muscle is supported by superfusion of metabolites. Second, if the preparation is too small, the fibers

along the dissection plane which inevitably are destroyed contribute a larger proportion to the overall size, hence less healthy muscle is able to generate force. In consideration for the upper and lower limits to muscle preparation size, we found that a 2 mm swath of the ADM was the optimum lateral thickness for ex vivo mechanical measurements.

A previous study addressed the differences in sex with respect to masticatory muscle function in rabbits utilizing *in situ* stimulation of specific motor units (9), where males exhibited faster and stronger twitch contractions. The isolated muscle preparation in the current study utilizes supramaximal stimulation, which recruits all muscle fibers regardless of motor unit innervation. The resultant forces are a summation of all motor units in the muscle, and so single motor unit properties cannot be assessed. However, the past single motor unit measurements are similar to our findings (Table 1), and consistent with the shift toward faster fiber types and motor units in males.

Interestingly, the median fiber area in female ADM preparations was larger than that measured in males (Figure 4), suggesting that fiber area, *per se*, was not a contributing factor to the differences in rates of force generation and relaxation (Table I). Complete characterization of the mouse masseter fiber type and size observed a similar trend in the 2a fiber population in the same region (called the anterior intermediate masseter (5)). In that study, fiber diameter of MHC 2a fibers tended to be larger in females than males. However, other regions of masticatory muscle showed larger fiber diameter in males, regardless of fiber type. Indeed, in our own studies, we have observed larger fiber areas in the male superficial masseter, as well as other muscle groups in the hindlimbs of mice (data not shown). Whether the tendency of larger MHC 2a fibers in female mice reflects an adaptation in females to increase overall fiber area in this region to compensate for the diminished MHC 2b fiber population or the decrease in muscle length isn't clear, but it does not seem to prevail in other regions of the masseter or in other skeletal muscles of the body. The presence of MHC 2b in skeletal muscles from small mammals (including mice) is not recapitulated in most human muscles. However, the sexual dimorphism of fiber type distribution holds across all species studied thus far, supporting the utility of assessing trends in masticatory muscle function in this model.

Craniofacial muscles are involved in both genetic and idiopathic disorders. Facioscapulaohumeral muscular dystrophy (FSHD), a rare inheritable form of muscular dystrophy, begins with weakness in the facial muscles and progresses slowly to the rest of the body. It is not known why muscles of the head are the first to be involved. Temporomandibular disorders (TMD) are characterized by chronic pain in the temporomandibular joint (TMJ) and muscles of mastication. TMD encompasses primary diagnoses in the TMJ (arthrogenic) and the musculature (myogenic)(25). Myogenic TMD pathologies constitute at least 70% of known cases, and a disproportionate number of TMD patients who report myofascial pain are premenopausal women (26,27) suggesting that gender may be a contributing factor in the onset of TMD. However, a biological explanation for the propensity of females in the TMD population has not been identified. Because mouse models for both FSHD and TMD have been developed or are underway (28,29), the ability to measure the normal and pathological functional state of masticatory muscles could be utilized to assess potential therapies for these disorders.

In conclusion, sex does not affect maximum strength in the isolated murine masticatory muscles. However, sexual dimorphism of fiber type influences the rates of force generation and relaxation, which may impact rhythmic contraction patterns involved in mastication. Functional measurements of the muscles of mastication will provide a tool for preclinical evaluation of treatments for craniofacial muscle disorders.

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ABBREVIATIONS

ADM	anterior deep masseter
DAPI	4'-6-Diamidino-2-phenylindole
dP/dt	rate of force generation
FSHD	Facioscapularhumeral muscular dystrophy
Lo	optimum muscle length
MHC	myosin heavy chain
Po	maximum isometric tetanic force
Pt	maximum isometric twitch force
SP Force	Force per cross sectional area
TMD	temporomandibular disorders

TMJ

temporomandibular joint

1/2 Rel

relaxation time to 50% maximum force after stimulation

Figure 1.

Process of preparation of ADM for isolated muscle mechanics. **A.** Removal of superficial masseter occurs by cutting the tendon (*) to reveal the deep regions of the masseter. **B.** Region of the deep masseter (**) utilized for isolated muscle mechanical measurements. **C.** Suture attachment sites on the mandible and zygomatic process of the maxilla flank the region of muscle to be utilized for functional measurements.

Figure 2. Red fibers on the edge of the muscle cross section are filled with procion orange dye which has entered due to sarcolemmal damage. These fibers are discounted for function measurements. Nuclei are identified by DAPI to aid in the focusing of the section.

Figure 3.

Examples of isometric twitch (panel A) and maximum tetanic (panel B) force production in male and female ADM muscles. Twitch force tended to be lower in females but was not significantly different than twitch in males. Tetanic force did not differ with respect to sex. However, a slower rate of force production is apparent in the females.

Figure 4.

Fiber analysis of male and female ADM muscles. **A.** Box and whisker plots demonstrate that median fiber area was significantly larger in all female fibers, including MHC 2a and 2b positive fibers, compared to male fibers. Boxes identify the 25th percentile, median, and 75th percentile, and the end of the whiskers identify the minimum and maximum values for each data set. The maximum fiber area was in male muscle preparations, and consisted of large MHC 2b fibers. *, $P < 0.05$ for comparisons by the Mann-Whitney test. **B.** Mean values and standard deviations overlay the raw data for percent MHC 2b positive fibers in ADM muscles ($N=4$ muscles per group). The proportion of MHC 2b fibers was significantly greater in the male ADM muscles compared to females (by the Mann-Whitney test).

Table 1
Functional profile of murine anterior deep masseters

	Female	Male	P value
Dimensions			
Lo (mm)	7.02±0.11	7.61±0.10	0.001
Mass (mg)	13.78±0.79	14.28±0.73	0.649
CSA (mm ²)	1.85±0.10	1.77±0.10	0.573
Twitch			
Pt (mN)	47.66±5.50	58.31±4.48	0.179
TPT (msec)	41±2	40±1	0.618
Max dP/dt (g/sec)	378.38±36.22	461.14±16.73	0.088
1/2 Rel (msec)	24±1	25±1	0.241
twitch:tet	0.12±0.01*	0.16±0.01	0.019
Tetanus			
Po (mN)	390.66±31.14	368.06±20.27	0.585
Sp Force (N/cm ²)	21.21±0.79	21.18±0.88	0.424
Max dP/dt (g/sec)	381.76±32.77*	515.56±23.45	0.009
1/2 Rel (msec)	48±1	41±2	0.014

Mean ± SEM for N=8 ADM for each sex.

* p<0.05, significant difference between males and females.