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Voluntary biting behavior as a functional measure of orofacial pain in mice

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Abstract

Introduction: Pain-related behavior secondary to masticatory function can be assessed with the rodent bite force model. A reduction of the bite force has been shown to be related to pain associated with the masseter muscle and jaw activity, while an increase in bite force suggests improvement of muscle function and less pain. To evaluate the usefulness of the bite force measure in studying *long-lasting orofacial pain* we analyzed biting parameters during prolonged myofascial pain induced by ligation injury of the masseter muscle tendon (TL) in mice.

Methods: C57Bl/6 mice were habituated to bite at a pair of aluminum plates attached to a force displacement transducer. The transduced voltage signals were amplified and converted to force through calibration with a standard weight set. Voluntary biting behavior was recorded for 100 seconds/session and those with bite forces > 980 mN were analyzed. Nociception was also verified with von Frey, conditioned place avoidance (CPA) tests and mouse grimace scale. Persistent orofacial pain was induced with unilateral ligation of one tendon of the masseter muscle (TL).

Results: To reduce interference of random bites of smaller forces, the top 5 or 15 bite forces (BF5/15) were chosen as a measure of masticatory function and related to pain behavior. Both male and female mice exhibited similar BF5/15. For the first nascent test of all mice, mean bite force was significantly and positively correlated with the body weight. However, this correlation was less clear in the latter tests (2-8 w). TL induced a reduction of BF5/15 that peaked at 1 w and returned to the baseline within 3 w. The von Frey and CPA tests indicated that mechanical

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Author Contributions

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Conflict of interest

The authors declare no conflict of interests.

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allodynia/hyperalgesia persisted at the time when the BF had returned to the pre-injury level. Infusion of pain-relieving bone marrow stromal cells improved biting behavior in both male and female mice as shown by significantly increased BF5/15, compared to vehicle-treated mice.

Conclusions: Mouse voluntary biting behavior can be reliably measured and quantified with a simplified setup. The bite force showed an inverse relationship with the level of pain after TL and was improved by pain-relieving manipulations. However, the injury-induced reduction of bite force peaked early and did not parallel with other measures of nociception in the later phase of hyperalgesia. The results suggest that multiple factors such as the level of habituation, cognitive motive, physical status, and feeding drive may affect random voluntary biting and confound the biting parameters related to maintained hyperalgesia.

Keywords

Orofacial pain; Temporomandibular disorders; mesenchymal stromal cells; feeding; mastication; rodent

1. Introduction

The orofacial region offers a unique opportunity of using feeding-related behavior as a functional measure of pain. Feeding-related mastication or chewing involves the teeth, bones (the maxillae and mandibles), masticatory muscles (masseter, pterygoids, and temporalis) and the temporomandibular joint. The coordinated activities of these structures lead to optimal biting and food consumption and are indicative of the functional status of the masticatory system. It is commonly assumed that orofacial pain affects mastication-related activities, and as such, studies have explored the use of the biting parameters as a functional measure of pain, particularly for that associated with the temporomandibular joint disorders (TMJD). Clinical studies have shown changes in the bite force under orofacial pain conditions. In general, TMJD patients exhibited decreased bite force [1-3] or bite force endurance [4] and an increase in bite force is associated with reduced pain [5,6]. Experimentally-induced pain in humans leads to a reduction of the bite force [7]. The underlying mechanisms likely involve decreased motor neuronal activity in response to noxious stimulation [8,9].

Assessing nociception in the orofacial region has been challenging in animal studies. In addition to reflex measures, it is much desired to employ the rodent biting behavior to study the mechanisms and treatment of persistent orofacial pain. Ro and colleagues developed a method in rats that a target bite force can be achieved after training with a water reinforcement paradigm [10,11]. Induction of masseter muscle inflammation led to a reduction of the bite force, which was prevented with anti-inflammation agents [11]. Compared to rats, mice exhibit aggressive voluntary biting. Taking advantage of this phenomenon, Dolan et al. [12] designed the dolognawmeter that could be used to quantify the gnawing activity of the mouse. Orofacial pain, particularly oral cancer pain, was associated with a decrease in gnawing efficiency, which was reversed by morphine [12].

Several recent studies have employed voluntary bite force to assess complete Freund's adjuvant (CFA)-induced orofacial pain in mice [13-16]. It appears that a reduction of the bite

force occurred mainly within a few days after CFA-induced inflammatory hyperalgesia and the usefulness of the bite force in studying long-lasting pain has not been characterized and evaluated. To address this issue, we analyzed biting parameters during prolonged myofascial pain induced by ligation injury of the masseter muscle tendon (TL) in mice. Utilizing bone marrow stromal cells (BMSCs) that have been shown to attenuate persistent pain [17-20], we show that reduced bite force after TL was improved after infusion of BMSCs.

2. Materials and methods

2.1. Animals

Adult Male/Female C57BI/6 mice were used (Veterinary Resources, University of Maryland, Baltimore). Mice arrived at 8-w-old and the experiments started at one week after the arrival. Animals were kept under controlled conditions ($\approx 22^{\circ}\text{C}$), relative humidity 40-60%, 12h/12h light-dark cycles, and food and water *ad libitum*. All behavioral tests were conducted during the light cycle. Surgical procedures were performed under Ketamine/Xylazine anesthesia (100-150 mg/kg ketamine/10-16 mg/kg xylazine, i.p.). Ligation of the tendon of the mouse masseter muscle (TL) was adapted from an intraoral approach as described elsewhere in rats [21]. Briefly, on the left intraoral site, a 3-mm long incision was made posterior-anteriorly lateral to the gingivobuccal margin in the buccal mucosa, beginning immediately next to the first maxillary molar. The tendon of the masseter muscle was gently freed and tied with two chromic gut (4.0) ligatures, 2-mm apart. The Sham-operated animals received the same surgical procedure except that the tendon was not ligated. Animals were randomly assigned to the experimental groups (naïve, TL and Sham). All experiments were carried out in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) and approved by the Institutional Animal Care and Use Committee, University of Maryland School of Dentistry/Medicine. All behavioral tests were conducted under blind conditions. All von Frey and conditioned place avoidance (CPA) tests were conducted by female experimenters and bite force test was conducted by both male and female experimenters.

2.2. Measurement of biting behavior

Mouse voluntary biting was assessed as described [15]. A 60-ml plastic syringe was modified to form a cylindrical tube restrainer. The tip of the syringe was cut open to just allow the mouse to protrude its head out. The cutting edge was sanded smooth to avoid additional mechanical stimulation and discomfort. Mice were acclimated to enter the opening of the restrainer on the other end and protrude the head out of the restrainer. The plunger was reinserted into the syringe tube once the mouse had entered the tube to prevent backward movement. Mice were monitored throughout the entire period while they stayed inside the restrainer. Mice were released to the home cage if there were signs of discomfort or stress, such as vocalization, abnormal aggressive behavior, piloerection, diarrhea, and tachypnea. Once the animal was acclimated to comfortably protrude the head out of the restrainer, the syringe containing the mouse was held manually and moved slowly toward the biting head to initiate biting upon contact. The bite head consists of a pair of aluminum plates attached to a force-displacement transducer (Fig. 1A). The bite plates were customer-processed at the Machine Shop of the University of Maryland, Baltimore County and further

modified to fit the transducer (FT03, Grass Instruments). The transduced voltage signals were amplified and fed into Spike 2 via CED 1401 Plus and converted to force through calibration with a standard weight set. A customized interactive data capture script program was used to set up for data acquisition and analysis [10,11]. A 100-s period of voluntary biting behavior was collected per session and those with bite forces ≥ 980 mN (arbitrary cut off force) were analyzed.

2.3. Measurement of mechanical sensitivity

The mechanical sensitivity test of the orofacial region was adapted from rats [21,22]. Different from the rat, the mouse was habituated to be held entirely in the experimenter's hand wearing a leather work glove. In a well-lighted room, the shaded col within the palm of the glove appeared attractive to the mouse and facilitated habituation. Since mice are more mobile, a longer period of acclimation is often needed before formal testing. Care was taken to identify a false response such that head withdrawal was initiated before the probe-skin contact. A series of calibrated von Frey filaments were applied to the skin above the injured tendon. An active withdrawal of the head from the probing filament was defined as a response. Each von Frey filament was applied 5 times at intervals of 5-10 seconds. The response frequencies [(the number of responses/number of stimuli) $\times 100\%$] to a range of von Frey filament forces were determined and a stimulus-response frequency (S-R) curve plotted. After non-linear regression analysis, an EF_{50} value, defined as the effective von Frey filament force (g) that produces a 50% response frequency, was derived from the S-R curve (Prism, GraphPad) [23]. Tendon ligation leads to a leftward shift of the S-R curve and a reduction of EF_{50} , suggesting the development of mechanical hypersensitivity.

2.4. Measurement of conditioned place avoidance behavior (CPA)

For measurement of conditioned place avoidance behavior [24], animals were placed within a 42 (L) $\times$ 18 (W) $\times$ 18 (H) cm Plexiglas chamber. One half of the chamber was made by white plates (light area) and the other half was by black plates (dark area). During behavioral testing, animals were allowed unrestricted movement throughout the test chambers for the duration of a 30-min test period. Mice normally prefer to stay in the dark area. Testing began immediately with suprathreshold mechanical stimulation (117 mN von Frey monofilament) applied to the facial skin at 15-sec intervals. The mechanical stimulus was applied to the skin above the injured tendon when the animal was within the preferred dark area and to the facial skin on the non-injured side when the animal was within the non-preferred light side. Naïve animals were mechanically stimulated in the same manner as the injured groups. Based on the location of the animal at each 15-sec interval, the mean percentage of time spent in each side of the chamber was calculated for the entire test period. Staying in the preferred dark side is associated with an aversive and painful stimulus after TL and the injured mouse is forced to decide whether to remain there or leave to avoid the aversive stimulus. The tendency to move to and stay in the light area is a measure of the aversiveness of the stimulus.

2.5. Grimace scale [25,26]

Facial grimace scale was used to assess spontaneous pain after TL as described [15]. Briefly, the mice were placed in a cubicle (9 $\times$ 5 $\times$ 5 cm high), with transparent Plexiglas walls, a

ventilated metal shelf bottom, and an opaque middle wall that separated 2 cubicles and allowed the recording of 2 mice at a time. Two digital video cameras (Sony HDR-CX230/B High Definition Handycam Camcorder; Tokyo, Japan) were placed at a fixed distance from the cubicle, with one on each side of the cubicle to capture the faces of the mice. Animals were acclimated to the testing environment for 3 days (30 minutes per day) before the assessments. The mice were videotaped for 30 minutes for each session. Image extraction was performed by experimenters blinded to experimental conditions. Images containing a clear view of the entire face were manually captured every 3 minutes during the video recording (10 images per 30-minute session). For consistency in scoring, one experimenter scored all the images in a blinded manner. Five facial action units (AUs) were scored: orbital tightening, nose bulge, cheek bulge, ear position, and whisker change. Each AU was scored 0, 1, or 2 on the basis of criteria described previously [25]: “0”, an absence of the AU; “1”, a moderate appearance of the AU; and “2”, obvious detection of the AU. An initial score of each photograph was the average of the scores of 5 AUs, and a mean score was obtained from the 10 images, which was presumed to reflect the level of spontaneous pain.

2.6. Bone marrow stromal cell procedure

Bone marrow stromal cells (BMSCs) were obtained from donor Sprague-Dawley rats (Envigo-Harlan) as described [17]. The rats were euthanized with CO₂ and both ends of the tibiae, femurs and humerus were cut off by scissors. A syringe fitted with an 18-gauge needle was inserted into the shaft of the bone and bone marrow was flushed out with culture medium (alpha-modified Eagle medium, Gibco, Carlsbad, CA, USA; 10% fetal bovine serum, Hyclone, Logan, UT, USA). The bone marrow was then mechanically dissociated, and the suspension passed through a 100-μm cell strainer to remove debris. The cells were incubated at 37°C in 5% CO₂ in tissue-culture flasks (100 × 200 mm) (Sarstedt, Nümbrecht, Germany), and non-adherent cells removed by replacing the medium. At day 7, when the cultures reached 80% confluence, the cells were washed with PBS and harvested. The cell numbers were calculated by the Hemacytometer. For systemic transfusion, 3 × 10⁵ cells in 0.1 ml PBS were slowly injected into one tail vein of the anesthetized mouse over a 2-minute period using a 30-gauge needle. The property of expanded cells was assessed by flow cytometry with conventional markers [17,19]. Flow cytometry analyses were performed at the University of Maryland Greenbaum Cancer Center Shared Flow Cytometry Facility.

2.7. Data analysis

The sample size was estimated by power analyses (G*Power version 3.1.9) and previous studies [15,16]. Data are presented as mean (95% confidence interval) for von Frey filament force data (EF₅₀) and mean ± S.E.M. for other data. Statistical comparisons were made by the use of analysis of variance (ANOVA). For unbalanced sample size, multiple regressions were performed, and Type III sum-of-squares was used to calculate P values (GraphPad Prism). The Tukey test was performed for *post-hoc* comparisons with Bonferroni correction. For animals that were subject to repeated testing, ANOVA with repeated measures was used. The Pearson product-moment correlation coefficient of determination (r^2) was determined between bite force and body weight. $P < 0.05$ was considered significant for all cases.

3. Results

3.1. Biting in naïve mice

We first characterized voluntary biting behavior in naïve mice. After acclimation, mice showed random biting activity upon contacting the bite plates. The bite was vertical and mainly involved incisors. The biting incidents were captured, transduced into a voltage signal, and displayed from the analysis script program, as shown in Fig. 1B. The overall bite forces exhibited unimodal distribution with the mode appears around 5,000 mN in both male and female mice (Fig. 2A).

To select a parameter for quantitative comparison, we compared mean bite forces of All bites (BF-All), Top 15 (BF-15) and Top 5 (BF-5) bites (Fig. 2B). The BF-All was the smallest among the three means ($p < 0.05$ - 0.001). Apparently, the smaller means of all bites were caused by smaller bite forces distributed to the left of the mode (Fig. 2A) that contained outliers at the low end of voluntary bite force [27]. Smaller bites represented random biting incidents that were seemingly without much effort and tended to confound the mean of the “maximal” bite force. Thus, it is inappropriate to use the mean of the overall bite force to compare biting activity. The means of Top 5 and 15 bites were similar, although male showed a slightly higher Top 5 mean value. Even though voluntary, the top bite forces were likely produced by close-to-maximal effort and reflected the functional status of the masticatory apparatus. Either BF-5 or BF-15 could be used for the quantitative analysis of bite forces. There were no significant differences in bite force means (Fig. 2B) and the number of bites per session (Fig. 2C) between males and females. The baseline body weight was lighter in female mice ($p < 0.001$) (Fig. 2D).

3.2. Correlation between the bite force and body weight

The bite force is positively related to body weight in humans [28,29]. To use bite force as a measure of long-lasting pain, the factor of body weight change needs to be considered. We performed Pearson correlation analyses between the bite force and body weight of the mouse. For the first (nascent) test of all mice, mean bite force was significantly and positively correlated with the body weight. Fig. 3A shows a correlation of the first BF-5 with starting body weight in male and female mice. Significant positive correlations between body weight and BF-15/BF-All were also observed (not shown).

We next asked whether the positive correlation between the body weight and bite force could be maintained over time after repeated testing. Surprisingly, the correlation was generally lost in latter tests (2-8 w) (Table 1). Even with smaller samples, male mice showed positive and moderate body weight-bite force correlation in the first week ($r^2 = 0.48$, $p = 0.04$). Female mice also showed a strong trend of positive correlation in the first week ($r^2 = 0.36$, $p = 0.07$). However, in the next 7-week testing, only males at week 3 showed a positive correlation ($r^2 = 0.5$, $p = 0.03$). Mean bite forces fluctuated and tended to be above the baseline level during repeated testing, although most changes were not statistically significant (Fig. 3B). The body weight progressively increased over time, but the bite force did not follow the same pattern (Fig. 3B). A general observation is that some animals gained experience with the test and exerted greater effort to bite in latter tests. Female mice weighed less than males,

but we did not see a difference in the bite force between female and male mice (Fig. 2). Thus, additional factors may confound the weight-force correlation observed in the initial test.

3.3. Effect of injury on bite force

After taking the baseline values, the mice were divided into a tendon ligation group and naïve group. The tendon ligation groups' anterior superficial part of the masseter tendon was ligated unilaterally as described [21]. Tendon injury induces mechanical pain hypersensitivity that lasts for months. The bite force was tested weekly after injury. Naïve mice were tested in parallel. Except at 1 w after injury, the bite forces exhibited similar unimodal distribution as shown in Fig. 2A (Fig. 4). Tendon injury produced a clear shift of the mode to the left (Fig. 4B, middle) at the one-week time point, indicating a lack of bites with greater effort. Examples of bite-force measurements are shown in Fig. 5. Consistent with the histograms, tendon injury induced an apparent decrease in biting magnitude in both sexes.

The bite forces were further quantitatively compared between naïve and injured mice. Following TL, the greatest reduction in bite force (BF-15) occurred at 1 w after ligation (Fig. 6A-C). The BF-15 of TL mice started to increase at 2 w and did not differ statistically from the baseline at 4 w (male) and 2 w (female). However, compared to naïve mice, both males and females showed significant differences at multiple points at 1-7 w after injury (Fig. 6A, B), likely a result of the above-baseline shift of bite force in naïve mice after repeated testing. Normalization of BF-15 to the percent of baseline showed that about 60% reduction occurred at 1 w and BF-15 of all later times were within 20% of the baseline (Fig 6C). Body weight slightly reduced at 1 w and showed constant grow afterward in male mice (Fig. 6A, Right). Body weight of female mice reduced at 1 w after TL, grew slowly toward the baseline in the following weeks and reached the baseline weight at 8 w after injury (Fig. 6B, Right). Thus, TL appeared to have a greater effect on the growth of females. Two-way ANOVA did not reveal a difference in body weight between naïve and injured mice. There were no differences in the number of biting per session between naïve and TL mice.

Thus, the most dramatic effect of TL on bite force was seen at one week after injury (Figs. 4-6). Using the von Frey method in rats, TL-induced mechanical hypersensitivity was maintained at a constant low level throughout the 8-week observation period [21]. We verified this with a group of female mice. As shown in Fig. 6D, a significant reduction of EF₅₀, a measure of mechanical allodynia and hyperalgesia, was maintained through 8 weeks after injury. Conditioned place avoidance test confirmed that TL mice spent a significant higher amount of time in the light area at 7 w after TL (Fig. 6E), indicating injury-related pain aversiveness. The mouse grimace scale scores also showed significant increases in a group of TL mice, compared to sham-operated animals (Fig. 6F), indicating the presence of spontaneous pain. These results suggest that the bite force is less sensitive in detecting long-lasting behavioral nociceptive hypersensitivity.

3.4. Effect of bone marrow stromal cells on bite force

We finally verified whether the bite force measure could be used to detect pain-relief. We have shown that transplantation of BMSCs produces long-lasting pain relief in TL rats [17-20]. Since BMSCs are able to avoid immune rejection likely due to a lack of major histocompatibility complex class II molecules and co-stimulatory surface markers for T-cell activation [30], we infused rat BMSCs to mice. Mice tolerate rat BMSCs and survive well [19]. From the temporal profile of TL-induced changes in bite force, we reasoned to examine the effect of BMSC in the first 15 days of injury. Mice were tested before TL and BMSCs were infused i.v. (3×10^5 cells in 0.1 ml PBS) at 1 d after TL. To avoid an effect of repeated testing, particularly after injury, mice were not tested before the injection of BMSCs, except the baseline test. As shown in Fig. 7A, BMSCs attenuated biting activity, compared to the vehicle-treated mouse at 2 d after BMSC infusion. The mean BF-15s were significantly larger at 2, 8 and 15 d after BMSC infusion in both male and female mice, compared to vehicle treatment (Fig. 7A, B). Von Frey responses and CPA (13 d post-BMSCs) were also improved after BMSC, as indicated by elevated EF_{50s} and decreased time in the light chamber (Fig. 7A, B, middle). There were no significant differences in body weight between BMSC and vehicle treatment.

4. Discussion

Here we performed quantitative and comparative analyses on the use of voluntary biting behavior as a functional measure of *persistent* orofacial nociception in mice.

4.1. Bite force-body weight correlation

The bite force is largely determined by the function of masticatory muscles. The thickness of the masseter is positively correlated with bite strength [31]. The bite force is also positively related to body weight in humans [28,29]. Thus, to use the bite force to assess lasting orofacial pain, the body weight might be a confounding factor since the loss in the masseter muscle mass parallels body weight loss [32]. We noted that the nascent bite force showed high-moderate correlation with the body weight, particularly when the body weight was over 30 g (Fig. 3A, Male). However, this correlation was less clear after repeated testing during an eight-week period. Another interesting observation was that age-matched females weighed significantly less than males, but their bite forces were similar to the males. Some human studies also did not see sex difference in bite force [5,33]. Female mice seem trickier in incising activity [34]. These results suggest that factors such as level of habituation to the test, motivation, physical status and sex may offset the effect of body weight on biting behavior. The biting behavior may also be affected by stress related to the use of a tube-restrainer [35,36]. Although the mice tested did not show signs of stress after habituation, the potential effect of restraint stress could not be completely ruled out. Additionally, the experimenter sex has been shown to have an effect on rodent nocifensive behavior. A male tester induces a stress response and reduced nocifensive behavior [37]. It is yet to be determined whether the bite force measure is also subject to modulation by the sex of the experimenter.

4.2. Injury, pain and bite force

Chewing is usually reduced in humans suffering from orofacial pain. In the rodent biting model, the existing pain conditions lead to reduced biting activity [10-13, 34,38,39]. Reduced biting may lead to inefficient feeding and affect growth, which may further reduce bite force. However, it is still unclear whether the bite force model can be used to assess lingering pain conditions. Inflammation of the TMJ or masseter muscle induces nociceptive hypersensitivity that lasts for days and weeks [22, 40,41]. Yet a decreased bite force was only shown in the first day or a few after injection of the inflammatory Freund's adjuvant into the TMJ or masseter [11,13-15]. A reduced chewing efficiency, as assessed with the dolognawmeter, was seen mainly at 10-hour after TMJ or masseter inflammation [12].

In the present study, a significant reduction of bite force peaked at 1 w and returned to the baseline level within 3 w after TL. The parallel von Frey and CAP behavior tests indicated that pain hypersensitivity persisted at times when the biting force had returned to the pre-injury level. These observations would be consistent with the view that feeding behavior is highly protected in rodents [42,43]. After TMJ or masseter muscle inflammation, animals are able to maintain their weight [21,44,45]. Using food intake as a measure of nociception, the meal duration, but not the amount of food consumption showed changes [45,46]. Mastication is adaptive to pain conditions, as evaluated in human subjects under monosodium glutamate-induced jaw muscle or TMJ pain [47,48]. It has been shown that there is re-organization of activity in "painful" muscle following injection of hypertonic saline into the masseter [49]. Clinical evidence indicates that although some TMJD patients did not show reduced bite force, they tend to avoid hard food [50,51]. Similarly, some incising parameters such as incising episodes can be maintained normal in mice with masseter hyperalgesia [52]. Foo and Mason [42] showed that in the brainstem pain-modulatory circuitry, pain-facilitatory neurons were inhibited and pain-inhibitory neurons were excited during feeding, thus providing a cellular mechanism that linking chewing activity with nociception. In fact, feeding following food-deprivation can induce pain inhibition that is endogenous opioid-mediated [53].

Another factor to be considered is the adaptation of the model that muscle activity is reduced to limit movement and protect the body from further injury [54]. Although the bite force appeared less sensitive in detecting long-lasting behavioral nociceptive hypersensitivity, we noted an intriguing difference between naïve and injured animals. Naïve mice showed apparently increased bite force during repeated weekly testing, which may be related to the habituation or adaptation to the test as training can improve biting efficiency [10,12]. Similarly, the maximum bite force in patients with complete dentures increases during repeated testing [55]; and repeated exercises of the masticatory muscles can reduce muscle pain and fatigue [56]. In patients of first bite syndrome, pain triggered by the first bite of a meal improves in the subsequent bites [57,58]. Apparently, the temporal adaptation of voluntary biting as well as the motive for feeding may confound the effect of injury on bite force. Thus, although the biting behavior is affected by painful orofacial structures, survival feeding drive and reorganization of the integrated feeding-sensory-motor neural circuitry may compete with painful conditions to maintain body homeostasis and confound the effect of injury on biting.

A limitation of the present study is that only bite force from unidirectional biting was used to assess biting behavior. In mice receiving an acidic saline injection into the masseter muscle, normal incising activity assessed with three-dimensional biting could be maintained during hyperalgesia, but the incising frequencies were lowered over a 3-w period [52].

4.3. The effect of pain-relieving BMSCs

Bone marrow stromal cells, a major type of mesenchymal stromal cells, have generated considerable interest as a candidate for cell-based therapy. Transplantation of human or rat BMSCs produces long-term attenuation of hyperalgesia/allodynia (antihyperalgesia) in rodents [17,59]. Assessed with the von Frey method and CPA behavioral test, we have shown previously that TL-induced pain hypersensitivity was attenuated by BMSCs [17-20]. Although the majority of systemic BMSCs are trapped in the lungs after infusion and stayed in the body for no more than a few weeks, they produce antihyperalgesia that lasts for a few months, involving immune interactions and activation of the endogenous opioids [19,60].

Dramatic reduction of bite force after orofacial injury is a functional measure of orofacial pain and the window of abnormal biting after TL allowed evaluation of the effect of pain-relieving manipulations. Consistent with other measures, BMSCs improved bite force in a two-week period after TL. These results are consistent with the literature that reduced biting is related to orofacial pain, which is sensitive to pain-relieving manipulations [5,11-14,57].

4.4. Sex differences in voluntary biting behavior

As there is sexual dimorphism in the kinetics and fiber composition in the masseter muscle [61-63], one would expect to see sex differences in the bite force. However, we did not find a difference in mean bite forces between the male and female mice under baseline and injured conditions. Similar observations have been reported [13; 52]. This seems against a positive correlation of the nascent bite force with body weight, considering lower body weight in age-matched female mice and a greater effect of injury on body weight in females [52, Present results]. However, we cannot exclude a potential sex difference in biting behavior since our bite force measure was derived from random voluntary behavior that may not always represent the real maximal effort. Widmer and Morris-Wiman [52] used the home-cage biting model and noted that the top 10% bite force was not different between the male and female mice, likely related to a fact that chewing food pellet does not require maximal effort. We also noted that the TL mice, particularly females, could alternate the healthy side to incise, which would compensate for the tenderness on the site of tendon injury. Female mice are able to develop a novel incising strategy to accommodate pain, while males do not do so as frequently [34]. There was also a significant difference in preferential incising direction between male and female mice [34]. Repetitive acidic saline injections into the masseter muscle induce decreased incising frequencies and this effect lasts significantly longer in female compared to male mice [52].

4.5. Conclusions

Mouse voluntary biting behavior can be reliably measured and quantified with a simplified setup. The changes in bite force showed an inverse relationship with the level of pain after

orofacial injury and sensitive to pain-relieving manipulations, thus providing a functional measure of orofacial pain. However, the injury-induced reduction of bite force peaked early after TL and did not parallel with other measures of nociception in the later phase of hyperalgesia. The results suggest that multiple factors such as the level of habituation, cognitive motive, physical status, and feeding drive may affect random voluntary biting and confound the biting parameters related to persistent pain.

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Highlights

- Mouse voluntary biting can detect orofacial pain at the early phase of injury.
- Mouse voluntary biting seemed less sensitive in detecting lingering pain after injury.
- Infusion of bone marrow stromal cells improved biting behavior in injured mice.
- Repeated testing may confound quantified biting behavior in mice.
- Multiple factors may confound the biting parameters related to persistent pain.

Fig. 1. Bite force measurement in mice. **A.** Schematic illustration of the experimental set-up. The bite head (upper left) consists of two aluminum plates. The metal plates were custom-made and processed to fit the force-displacement transducer. The parameters of the plates are detailed (lower left). The flowchart (right) shows the flow of the transduced force signal. **B.** An example of screen views displaying traces of biting activity, shown as downward deflections of voltage signals. The dashed line with a number below was related to the calibration with a standard weight set and used to derive bite force.

Fig. 2.

General comparison of biting behavior between male and female mice. **A.** Histograms show the distribution of baseline bite forces. **B.** Mean bite force values of All, Top 15 and Top 5 bites. *, $p < 0.05$; ***, $p < 0.001$, vs. respective All. #, $p < 0.05$, vs. male top 5. ANOVA and *post-hoc* comparisons. **C.** The mean number of bites per session. **D.** Body weight (BW) comparison. ***, $p < 0.001$, vs. males.

Fig. 3.

Correlation of mean bite force with body weight. **A.** Mean top 5 bites force (BF-5) at baseline positively and significantly correlated with body weight in both male and female mice. **B.** Mean bite forces (circles and squares) over an 8-w period in naïve mice. Corresponding body weight (BW, diamonds) is plotted below with Y-axis on the right. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$, vs. respective week 1 values.

Fig. 4.

Bite force distribution in naïve and masseter muscle tendon-injured (TL) mice. **A.**

Histograms illustrating the distribution of bite forces in naïve mice. **B.** Bite force histograms of TL mice. Note a leftward shift of the mode at 1 w after TL.

Fig. 5. Examples of transduced voltage signals from bite-force recordings. Each trace shows bite-force recording in a 100-s session. Note reduced biting activity at 1 w after tendon ligation in both male (**A**) and female (**B**) mice.

Fig. 6.

The effect of tendon ligation (TL) on bite force and other measures. **A, B.** Changes in BF-15 and body weight in TL and naïve male (A) and female (B) mice. Data of naïve mice were shown in Fig. 3B and re-plotted here for comparison. **C.** Comparison of BF-15 as % of baseline (dashed line). **D.** TL-induced mechanical hypersensitivity in female mice. **E.** Conditioned place avoidance (CPA) test confirmed TL-induced aversive behavior. Compared to Naïve mice, the percentage of time mice spent within the light area was increased after TL (7w). **F.** Mouse grimace scores (MGS). *, $p < 0.05$, **, $p < 0.01$, *** $p < 0.001$, vs.

respective baseline (week 0) or naïve (E). ##, $p < 0.01$, ###, $p < 0.001$, vs. naïve (A, B) or Sham (F). ANOVA with *post-hoc* comparisons.

A. 2d post-BMSCs **B. Male** **C. Female** **Fig. 7.**

The effect of BMSCs on bite force of TL mice. **A.** Examples of transduced voltage signals from bite-force recordings. Each trace shows bite-force recording in a 100-s session. Compared to treatment with BMSCs, there was reduced biting activity in veh-treated mouse at 3d after tendon ligation. **B. C.** Summary of the effects of BMSCs on bite force, von Frey response, CPA and body weight. CPA was measured at 13 d post-BMSCs. *, $p < 0.05$, ***, $p < 0.001$, BMSC vs. Veh.

Table 1.

Correlation analysis between the body weight and bite force (BF-5) during an 8-week period.

Week	Male (n = 9)		Female (n =10)	
	r ²	p	r ²	p
1	0.48	0.04	0.36	0.07
2	0.20	0.22	0.12	0.34
3	0.50	0.03	0	0.85
4	0.02	0.73	0	0.98
5	0	0.97	0.12	0.32
6	0.37	0.06	0.10	0.38
7	0.16	0.28	0.08	0.42
8	0.22	0.20	0.18	0.23