Mechanism of motor coordination of masseter and temporalis muscles for increased masticatory efficiency in mice

Running head: Motor coordination of masseter and temporalis muscles

Article category: Original research

TOMOKO YOSHIMI[1], YOSHIYUKI KOGA[1], AYA NAKAMURA[1], AYUMI FUJISHITA[1], HARUKA KOHARA[1], EMI MORIUCHI[1], KEIKO YOSHIMI[2], CHI-YANG TSAI[3], NORIAKI YOSHIDA[1]

[1]Department of Orthodontics and Dentofacial Orthopedics, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, Japan
[2]Yoshimi Skin Clinic
[3]Division of Orthodontics, Department of Dentistry, Taipei Medical University Hospital, Taipei, Taiwan

Correspondence: Dr. Y. Koga, Department of Orthodontics and Dentofacial Orthopedics, Graduate School of Biomedical Sciences, Nagasaki University, 1-7-1 Sakamoto, Nagasaki, 852-8501, Japan.

E-mail: koga@nagasaki-u.ac.jp

Summary

The demand for the use of mice as animal models for elucidating the pathophysiologies and pathogeneses of oral motor disorders has been increasing in recent years, as more and more kinds of genetically modified mice that express functional disorders of the stomatognathic system become available. However, the fundamental characteristics of mouse jaw movements during mastication have yet to be fully elucidated. The purpose of this study was to investigate the roles of the masseter and temporalis muscles, and the mechanisms of motor coordination of these muscles for increasing masticatory efficiency in the closing phase in mice. Twenty-two male Jcl:ICR mice were divided into control (n = 8), masseter hypofunction (n = 7), and temporalis hypofunction groups (n = 7). Botulinum neurotoxin type A (BoNT⁄A) was used to induce muscle hypofunction. The masticatory movement path in the horizontal direction during the occlusal phase became unstable after BoNT⁄A injection into the masseter muscle. BoNT⁄A injection into the temporalis muscle decreased antero-posterior excursion of the late-closing phase corresponding to the power phase of the chewing cycle. These results suggest that the masseter plays an important role in stabilizing the grinding path, where the food bolus is ground by sliding the posterior teeth from back to front during the occlusal phase. The temporalis plays a major role in retracting the mandible more posteriorly in the early phase of closing, extending the grinding path. Masticatory efficiency is thus increased based on the coordination of activities by the masseter and temporalis muscles.

Keywords: mastication, electromyography, jaw relation record, masseter muscle, temporal muscle, botulinum toxin type A

Introduction

Mastication is the common rhythmical behavior of crushing and grinding food between the upper and lower teeth, and mixing this food with saliva to form a swallowable food bolus in mammals. This fundamental function for the conservation of life is performed with many organs involved in the complex ingestive process.[1] Various animal models have been used in attempts to elucidate the coordination of muscle activities relevant to mastication and the central and peripheral control mechanisms.[2,3] As marked progress in molecular biology has been made in recent years, several genetically modified mouse models for studying oral motor disorders have been developed,[4,5] and could be expected to enhance our understanding of the pathophysiologies and pathogeneses involved. However, basic characteristics of jaw movements during mastication in mice are not yet fully understood. In a previous study, we developed a system for measuring three-dimensional jaw movements with simultaneous recording of jaw muscle activities in mice.[6] We found interesting features in the jaw movement trajectories of mice that are characteristically different from those of human, particularly in the closing phase.[7] That is, paths of jaw opening and closing diverge widely in the sagittal plane, and the mandible traces a more posterior path on closing than on opening. Utsumi *et al.*[8] reported that the temporalis plays an important role in retracting the mandible during the early-closing phase, then the masseter would pull the mandible anteriorly during the late-closing phase. They also suggested that antero-posterior discrepancies between the jaw opening and closing paths in the sagittal plane are formed by regulation of the motor coordination between the masseter and temporalis muscles,

contributing to increased masticatory performance. The food bolus is thus considered to be efficiently ground by sliding posterior teeth from back to front in the occlusal phase in mice. We therefore focused on the functional contribution of the masseter and temporalis to an establishment of proper masticatory movements in this study.

To clarify the mechanisms underlying the coordination of masseter and temporalis muscle activities and roles of individual muscles in greater detail, evaluating alterations in the pattern of jaw movements by causing hypofunction in specific muscles may be useful. In previous studies, commonly used methods to reduce masticatory function or induce muscle atrophy have included myectomy and denervation of the muscle.[9] However, these methods involve some critical problems, such as causing scar tissue formation, and consequently a potential inhibition of craniofacial growth.[10] Several recent studies have induced masticatory hypofunction using botulinum neurotoxin type A (BoNT/A) injection to cause temporary nerve blockade.[11,12] BoNT/A injection into a specific muscle is considered to lead to temporal denervation, thereby inducing specific muscular atrophy without irreversible soft tissue scarring or the change of the surrounding muscular environment. When BoNT/A is injected into the masseter or temporalis muscle selectively in mice, the pattern of jaw movements or muscle activities would be altered due to masticatory hypofunction, which could identify the functions of those muscles during mastication.

The purpose of the present study was to clarify the roles of the two major jaw-closing muscles and to test the hypothesis that motor coordination of the masseter and temporalis muscles could represent a

key determinant of increased masticatory efficiency in the closing phase in mice.

Material and methods

The experimental protocol of this study was approved by the Animal Welfare Committee of Nagasaki University based on the Animal Care Standards of this institution (approval no. 1504091216, 2015). Every possible effort was taken to minimize animal suffering.

Experimental animals

Twenty-two male Jcl:ICR mice (Clea, Tokyo, Japan) were used. At 15 weeks old, mice were randomly divided into three groups that included a control group (n = 8) in which sterile 0.9% sodium chloride solution (saline), a masseter-hypofunction group (n = 7), and a temporalis-hypofunction group (n = 7), in which BoNT⁄A was injected into bilateral masseter and temporalis muscles, respectively. Animals were housed in plastic cages and provided with ad libitum access to water and hard pellet chow (CE-2; Clea, Tokyo, Japan).

Surgical preparation

Animals were anesthetized by intra-peritoneal injection of 5:1.5:3.5 ketamine (Ketaral; SankyoYell, Tokyo, Japan), xylazine (Selactar 2%; Bayer Health-care, Osaka, Japan), and 0.9% sodium chloride solution. For electromyography (EMG) recordings, bipolar electrodes consisting of teflon-coated

stainless steel wires with 2-mm exposed tips and 1-mm interpolar distance were implanted bilaterally into the masseter and temporalis muscles. For measuring jaw movements, four sensors and a target magnet were bonded respectively to parietal bones and the lower surface of the mandible using 4-META resin (Sun Medical, Moriyama, Japan). The sensor unit detected the magnet's displacement as jaw movement.

Injection of BoNT⁄A

After surgical preparation, the mice were allowed to recover for 3 days. To paralyze the masticatory muscles, we used BoNT⁄A (Botox Vista®; Allergan Pharmaceuticals, Irvine, CA). Mice were anesthetized by an intra-peritoneal injection of 5:1.5:3.5 ketamine, xylazine, and 0.9% sodium chloride solution before injection of BoNT⁄A. Each vial of 50 units of BoNT⁄A was then reconstituted with 5.0 ml of normal saline solution, yielding a preparation of 10 units/ml.

Appropriate volume and dose of BoNT⁄A were determined beforehand. That is, povidone-iodine was injected into muscles instead of BoNT/A as a marker for assessing the extent of the spread to determine the specific volume, wherein the injected solution could spread all over the target muscle and did not overflow into other muscles. Then, the specific dose of BoNT/A, which decreased the area of muscle activity (integrated EMG) by approximately two-thirds of the original EMG before its injection, was determined.

BoNT/A of 0.1 unit (0.01 ml), which was determined by the above-mentioned method, was injected into

a single point for each muscle on both sides. Figure 1 shows a lateral view of the two major jaw-closing muscles in the mouse. We referred to a previous study for injection sites.[11]

Functional recordings

Three-dimensional jaw movements and EMG of the masseter and temporalis muscles were recorded twice, before and after BoNT/A injections. According to the previous study[13], recording muscle activity in a stable manner for longer than a week after the placement of electrodes was difficult, since muscles are extremely thin in mice. Therefore, the first recordings were performed 3 days after surgical preparation (before BoNT/A injections), and the second recordings were performed the day after BoNT/A injections. We then compared jaw movements and muscle activities before and after BoNT/A injections.

Both three-dimensional jaw movements and EMG were recorded while animals were chewing ball-shaped hard pellet chow (diameter, 3 mm). Signals were amplified with AC amplifiers and stored in a computer memory through a 12-bit A/D converter. The sampling rate of recordings was fixed at 2000 Hz. This methodology has been described in detail in previously published articles.[6,7]

Data analysis

Figure 2 lists the parameters used to analyze jaw movements. Mean values for each parameter were calculated from 10 chewing cycles for each animal. In a previous study,[8] we determined that jaw

closure from the occlusal view could be divided into two phases: early-closing and late-closing (Fig. 2). The late-closing phase is considered the occlusal phase of the chewing cycle and the time when most of the food grinding and possibly some tooth contact takes place. EMG activities in the masseter and temporalis muscles were analyzed in terms of burst duration and area (integrated EMG). Spike 2 software (Cambridge Electronic Design, Cambridge, UK) was used to facilitate waveform analysis.

Statistical analysis

Significant differences between the three groups were determined by two-way repeated-measures analysis of variance (ANOVA), followed by Tukey's multiple-comparisons *post hoc* test, with paired *t*-testing used to examine differences between values before and after BoNT⁄A injections. Values of $P < 0.05$ were considered statistically significant. All values are displayed as means ± standard deviation of the mean.

Results

Figure 3 shows examples of jaw movements in the vertical, horizontal, and antero-posterior directions, and EMG of the masseter and temporalis muscles during mastication before and after BoNT⁄A injections for the control, masseter-hypofunction, and temporalis-hypofunction groups.

Jaw muscle activity

Comparison of muscle activities between the three groups demonstrated that the area of masseter activities after BoNT/A injection was significantly smaller, and the duration of masseter activities tended to be shorter (although no significant difference was present) in the masseter-hypofunction group than in the control group (Table 1). Similarly, area and duration of temporalis activities were significantly smaller and shorter, respectively, in the temporalis-hypofunction group than in the control group and masseter-hypofunction group. A significant difference in the area of masseter activities was also evident between the temporalis-hypofunction and control groups.

Comparison of muscle activities before and after BoNT/A injection showed that both the area and duration of masseter muscle activities in the masseter-hypofunction group and those of temporalis muscle activities in the temporalis-hypofunction group were significantly decreased after BoNT/A injection (Table 2). The area of the masseter muscle activities was also significantly smaller in the temporalis-hypofunction group.

Jaw movements

Three-dimensional jaw movement trajectories during pellet-chewing were reconstructed in two dimensions by projection onto the sagittal, frontal, and occlusal planes (Fig. 4).

Comparison of jaw movement parameters between the three groups showed that excursion and duration of the late-closing phase were significantly smaller and shorter, respectively, and also antero-posterior divergence was smaller, in the temporalis-hypofunction group than in the control

and masseter-hypofunction groups (Table 1). Lateral excursion was significantly smaller in the temporalis-hypofunction group than in the masseter-hypofunction group. Anterior dispersion was significantly larger in the masseter-hypofunction group than in the control and temporalis hypofunction groups.

Comparison of jaw movement parameters before and after BoNT⁄A injection demonstrated that anterior dispersion was significantly increased in the masseter-hypofunction group, while gape size was decreased after BoNT⁄A injection (Table 2). Significant decreases in excursion of the late-closing phase and antero-posterior divergence were observed in the temporalis-hypofunction group after BoNT⁄A injection, compared to before injection. The lateral excursion was significantly reduced following BoNT⁄A injection into the temporalis muscle.

Discussion

Effect of BoNT⁄A injection on muscle activity

Regarding the specific volume and dose of BoNT/A for inducing muscular hypofunction, the volume of 0.01 ml was considered to be appropriate, since injected solution was spread all over the muscle, and did not overflow into other muscles when povidone-iodine was used as a marker. The effect of BoNT/A injection was thus confirmed to be confined to within the target muscles. To determine the appropriate dose of BoNT/A for paralyzing muscles in the present study, we defined muscular hypofunction as a decrease in the area of muscle activity (integrated EMG) by approximately two-thirds of the original

EMG before BoNT/A injection. It was considered that the dose of 0.1 unit could sufficiently paralyze each muscle, since the area of muscle activity significantly decreased after BoNT/A injection.

To investigate the effects of BoNT⁄A injection on muscle activity and to confirm whether hypofunction of the masseter or temporalis muscles could be effectively induced, we compared results obtained from the three groups and those before and after injections. As a result, both area and duration of masseter muscle activities in the masseter-hypofunction group and those of temporalis muscle activities in the temporalis-hypofunction group were significantly decreased after BoNT⁄A injections as compared to before injections. These findings were in good agreement with a previous study that reported significantly decreased masseter muscle activities after BoNT⁄A injection into unilateral masseter muscles in rats.[14] BoNT⁄A injection was considered to cause obvious paralysis of the muscles, and thus induced hypofunction, since the blockade of acetylcholine release from the nerve terminal at the neuromuscular junction prevents muscle contraction.[15, 16]

Lee et al.[17] reported that neither hypertrophy nor increased electromyographic activity of the temporalis muscle were seen as mechanisms to compensate for atrophy of the masseter muscles after BoNT/A injection in a chronic study. Likewise, the present study using a mouse model of acute hypofunction found that the masseter and temporalis muscles do not compensate for the atrophy of each muscle.

On the other hand, Rafferty et al.[18] reported that the medial pterygoid muscle compensated at least in part for the BoNT/A-paralyzed masseter muscle. The medial pterygoid muscle may thus have partially compensated for masseter hypofunction in our study, but was not considered able to achieve full

compensation, since a significant difference in jaw movement trajectory was seen between before and after BoNT/A injection into the masseter.

As for foodstuffs, this study used only hard pellet chow, on which the mice had been raised, as test food because we wanted to compare jaw movements and muscle activity before and after BoNT/A injection under comparable natural conditions. Our previous study reported specific changes in jaw movement and muscle activity during chewing of foods with different hardness, providing further evidence that the output of the masticatory central pattern generator may be somewhat modulated by changes to sensory inputs.[8] If sensory inputs from muscle spindles are affected by BoNT/A injection, differences in results between foods with different textures will be smaller. If BoNT/A injection does not affect muscle spindles, a significant difference will be seen. An investigation of the effect of food texture on motor coordination of masticatory muscles will be the subject of future investigations.

Effect of BoNT⁄A injection on jaw movement

An interesting feature in jaw-movement trajectories was observed when BoNT⁄A was injected into the masseter muscle. That is, from the occlusal view, significantly larger dispersion was seen in the most anterior position in the lateral direction (anterior dispersion) after BoNT⁄A injection compared to before injection in the masseter-hypofunction group. On the other hand, no significant difference in anterior dispersion was seen between before and after BoNT⁄A injection in the temporalis-hypofunction group. By contracting mainly the masseter muscles in the late-closing phase, mice moved the jaw forward

along the grinding path, which is defined as a masticatory path in the occlusal phase where the food bolus was ground between the occlusal surfaces of the posterior teeth. At the end of the grinding path, anterior jaw movement diverged laterally to the right or left side toward the most anterior position from the occlusal view. Anterior dispersion could be used as an indicator of the stability of the grinding path in the occlusal phase. Previous studies have reported that BoNT⁄A induces not only paralysis of the muscles, but also reductions in sensory inputs from muscle spindles.[19-21] Instability of the grinding path in the late-closing phase of the chewing cycle may be attributable to inhibition of afferent muscle spindle activity caused by BoNT/A injection. Further study is necessary to verify whether muscle spindles are affected by BoNT/A, using methods such as the application of eperisone hydrochloride, which does not act directly on muscle spindles. Hiiemae[22] suggested that the masseter could stabilize the vertical and horizontal positions of the mandible in coordination with the temporalis, as well as produce a proper occlusal force from anatomical and mechanical perspectives. Coordination between the masseter and temporalis muscles may thus represent the main stabilizer of the lower jaw.

Mouse jaw movement trajectories showed a grinding pattern in the sagittal plane, unlike those of other animals or humans, which show a chopping pattern. That is, antero-posterior divergence was seen between the opening and closing paths, and the mandible traced a more posterior path in the closing phase than in the opening phase from the lateral view. Such antero-posterior discrepancy may be due to anatomical characteristics of mice. Sato[23] reported that the temporalis has a more posteriorly oriented force vector, while the masseter shows a more anteriorly oriented force vector. Since the onset

of temporalis muscle activity (at the beginning of the closing phase) was significantly earlier than the onset of masseter muscle activity (in the middle of the closing phase), the temporalis could easily retract the jaw more posteriorly in the early-closing phase.[8]

In the present study, a characteristic feature in jaw-movement trajectories was also observed after BoNT/A injection into the temporalis muscle. That is, with hypofunction of the temporalis, excursion of the late-closing phase and antero-posterior divergence were significantly smaller, and the late-closing phase was shorter than that before injection. These findings may indicate that hypofunction in the temporalis restricts posterior jaw movement, although mice could easily retract the jaw by activating the temporalis in the closing phase before BoNT/A injection.

It is, therefore, suggested that the temporalis may be of key importance for retracting the jaw more posteriorly in the early-closing phase to extend the grinding path in the occlusal phase, and consequently to increase the efficiency of grinding food with the posterior teeth. The masseter would then pull the mandible forward in the late-closing phase along the extended grinding path. At this time, the masseter would also play an important role in stabilizing the grinding path in the occlusal phase to optimize food grinding during the power stroke of mastication. The findings of the present study suggest that the coordination of the masseter and temporalis muscles is a key determinant of masticatory efficiency.

Major limitations currently exist to the long-term retention of bipolar electrodes in muscles, which are extremely thin in mice. As mice move or masticate after surgical preparation, settlement of electrodes

tends to be ineffective. That is, EMG became unstable, and the signal-to-noise ratio gradually decreased. In preliminary experiments, we recorded EMG on days 1, 3, and 5, after BoNT/A injections to investigate muscle activities on each day. As a result, signal-to-noise ratio on day 3 or 5 was determined to be low, although clear EMG was recorded on day one. For this reason, we used EMG values recorded on day 1 after BoNT/A injection. Methods of inserting electrodes or the design of electrodes themselves must be improved before long-term EMG can be reliably achieved. Such advances are prerequisite for establishing animal models of chronic muscle hypofunction and investigating the processes of compensation for muscles over time in the near future.

Acknowledgements

This study was supported in part by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan (grant nos. 16K15840, 16K20649, 15H05050, and 15K20599). None of the authors have any conflicts of interests to declare.

References

[1] Nakamura Y, Katakura N. Generation of masticatory rhythm in the brainstem. *Neurosci Res*. 1995; 23: 1-19.

[2] Dellow PG, Lund JP. Evidence for central timing of rhythmical mastication. *J Physiol*. 1971; 215: 1-13.

[3] Nakamura Y, Kubo Y, Nozaki S, Takatori M. Cortically induced masticatory rhythm and its modification by tonic peripheral inputs in immobilized cats. *Bull Tokyo Med Dent Univ*. 1976; 23: 101-107.

[4] Okayasu I, Yamada Y, Maeda T, Yoshida N, Koga Y, Oi K. The involvement of brain-derived neurotrophic factor in the pattern generator of mastication. *Brain Res*. 2004; 1016: 40-47.

[5] Sanefuji K, Zeredo JL, Kurose M, Tanaka M, Koga Y, Yamada Y, Yoshida N. Possible effects of periodontal inputs on the masticatory function. *J Jpn Soc Stomatognath Funct.* 2008; 14: 89-95.

[6] Koga Y, Yoshida N, Kobayashi K, Okayasu I, Yamada Y. Development of a three-dimensional jaw-tracking system implanted in the freely moving mouse. *Med Eng Phys*. 2001; 23: 201-206.

[7] Okayasu I, Yamada Y, Kohno S, Yoshida N. New animal model for studying mastication in oral motor disorders. *J Dent Res*. 2003; 82: 318-321.

[8] Utsumi D, Nakamura A, Matsuo K, Zeredo JL, Koga Y, Yoshida N. Motor coordination of masseter and temporalis muscle during mastication in mice. *Int J Stomatl Occl Med*. 2011; 3: 187-194.

[9] Guelinckx P, Dechow PC, Vanrusselt R, Carlson DS. Adaptations in the temporalis muscles of rabbits after masseter muscle removal. *J Dent Res*. 1986; 65: 1294-1299.

[10] Gardner DE, Luschei ES, Joondeph DR. Alterations in the facial skeleton of the guinea pig following a lesion of the trigeminal motor nucleus. *Am J Orthod*. 1980; 78: 66-80.

[11] Tsai CY, Lin YC, Su B, Yang LY, Chiu WC. Masseter muscle fibre changes following reduction of masticatory function. *Int J Oral Maxillofac Surg*. 2012; 41: 394-399.

[12] Tsai CY, Yang LY, Chen KT, Chiu WC. The influence of masticatory hypofunction on developing rat craniofacial structure. *Int J Oral Maxillofac Surg*. 2010; 39: 593-598.

[13] Fujishita A, Koga Y, Utsumi D, Nakamura A, Yoshimi T, Yoshida N. Effects of feeding a soft diet and subsequent rehabilitation on the development of the masticatory function. *J Oral Rehabil.* 2015; 42: 266-274.

[14] Tsai CY, Lei YY, Yang LY, Chiu WC. Changes of masseter muscle activity following injection of botulinum toxin type A in adult rats. *Orthod Craniofac Res*. 2015; 18: 202-211.

[15] Tremaine AM, McCullough JL. Botulinum toxin type A for the management of glabellar rhytids. *Clin Cosmet Investig Dermatol*. 2010; 3: 15-23.

[16] Blasi J, Chapman ER, Link E, Binz T, Yamasaki S, De Camilli P, Südhof TC, Niemann H, Jahn R. Botulinum neurotoxin A selectively cleaves the synaptic protein SNAP-25. *Nature*. 1993; 365: 160-163.

[17] Lee CJ, Kim SG, Kim YJ, Han JY, Choi SH, Lee SI. Electrophysiologic change and facial contour following botulinum toxin A injection in square faces. *Plast Reconstr Surg*. 2007;120: 769-778.

[18] Rafferty KL, Liu ZJ, Ye W, Navarrete AL, Nguyen TT, Salamati A, Herring SW. Botulinum toxin in masticatory muscles: short- and long-term effects on muscle, bone, and craniofacial function in adult rabbits. *Bone*. 2012; 50: 651-662.

[19] Rosales RL, Dressler D. On muscle spindles, dystonia and botulinum toxin. *Eur J Neurol*. 2010; 17 Suppl 1: 71-80.

[20] Rosales RL, Arimura K, Takenaga S, Osame M. Extrafusal and intrafusal muscle effects in experimental botulinum toxin-A injection. *Muscle Nerve*. 1996; 19: 498-496.

[21] Filippi GM, Errico P, Santarelli R, Bagolini B, Manni E. Botulinum A toxin effects on rat jaw muscle spindles. *Acta Otolaryngol*. 1993; 113: 400-404.

[22] Hiiemae K. The structure and function of the jaw muscles in the rat (*Rattus norvegicus* L.). III. The mechanics of the muscles. *Zool J Linnean Soc.* 1971: 50: 111-132.

[23] Satoh K. Comparative functional morphology of mandibular forward movement during mastication of two murid rodents, *Apodemus speciosus* (Murinae) and *Clethrionomys rufocanus* (Arvicolinae). *J Morphol*. 1997; 231: 131-142.

Tables

Table 1. Comparison of jaw movements, cycle durations and muscle activity between experimental groups before and after BoNT/A injection.

Variables	Control group		Mas-hypofunction group		Temp-hypofunction group		Significance, *P*		
	before injection	after injection	before injection	after injection	before injection	after injection	control vs Mas-hypofunction	control vs Temp-hypofunction	Mas- vs Temp-hypofunction
Jaw movements (μm)									
1. GAPE	1726 ± 230	1694 ± 355	2207 ± 340	1955 ± 384	1938 ± 337	1785 ± 359	0.442	0.701	0.828
2. L-CL excursion	943.7 ± 295.2	1046.4 ± 190.9	1144.5 ± 226.7	1136.5 ± 344.1	1086.7 ± 168.6	626.8 ± 219.1	0.939	0.001	0.003
3. Antero-posterior divergence	637 ± 141	715 ± 109	798 ± 159	833 ± 142	773 ± 178	507 ± 216	0.943	0.006	0.002
4. Lateral excursion	1500 ± 332	1612 ± 183	1463 ± 306	1837 ± 602	1485 ± 276	1128 ± 284	0.433	0.063	0.006
5. Anterior dispersion	146 ± 30	155 ± 63	104 ± 34	233 ± 78	112 ± 40	109 ± 24	0.004	0.897	< 0.001
Cycle duration (ms)									
6. TC	253.7 ± 29.4	267.0 ± 83.4	240.6 ± 27.8	275.9 ± 27.8	227.9 ± 31.5	229.1 ± 43.5	0.869	0.777	0.478
7. OP	140.7 ± 25.8	151.4 ± 63.4	134.6 ± 19.7	152.6 ± 30.3	124.3 ± 24.1	122.5 ± 23.6	0.978	0.730	0.615
8. CL	112.9 ± 10.5	115.6 ± 22.2	106.0 ± 15.9	123.3 ± 13.0	103.7 ± 15.3	106.6 ± 25.0	0.478	0.972	0.365
9. E-CL	60.2 ± 85.9	59.7 ± 12.9	62.8 ± 9.0	65.6 ± 10.9	56.0 ± 10.6	70.5 ± 15.6	0.827	0.058	0.200
10. L-CL	52.7 ± 15.1	55.9 ± 12.4	43.1 ± 13.8	56.1 ± 13.0	47.7 ± 10.1	36.1 ± 12.9	0.912	0.046	0.023
Masseter muscle activity									
11. EMG duration (ms)	60.7 ± 8.8	63.7 ± 12.1	58.0 ± 8.4	43.1 ± 10.7	56.7 ± 10.8	52.5 ± 9.8	0.056	0.389	0.522
12. EMG area (AD per units)	0.0088 ± 0.0040	0.0099 ± 0.0019	0.0079 ± 0.0022	0.0024 ± 0.0009	0.0091 ± 0.0028	0.0063 ± 0.0030	< 0.001	0.009	0.017
Temporalis muscle activity									
13. EMG duration (ms)	79.8 ± 8.2	83.3 ± 18.4	88.3 ± 14.7	91.4 ± 15.7	74.8 ± 10.7	46.5 ± 26.1	0.977	0.019	0.026
14. EMG area (AD per units)	0.0069 ± 0.0025	0.0072 ± 0.0031	0.0061 ± 0.0022	0.0056 ± 0.0019	0.0057 ± 0.0016	0.0021 ± 0.005	0.519	0.002	0.027

Differences between the three experimental groups (control, masseter-hypofunction, temporalis-hypofunction) were investigated using two-way repeated-measures ANOVA. Dependent variable represents any post outcome and independent variable represents any pre outcome and group. We performed multiple comparisons using the Tukey *t* test. $P < 0.05$

Table 1. Comparison of jaw movements, cycle durations and muscle activity between experimental groups before and after BoNT/A injection.

Differences between the three experimental groups (control, masseter-hypofunction, temporalis-hypofunction) were investigated using two-way repeated-measures ANOVA. Dependent variable represents any post outcome and independent variable represents any pre outcome and group. We performed multiple comparisons using the Tukey *t* test. $P < 0.05$

Table 2. Comparison of jaw movements, cycle durations and muscle activity before and after BoNT/A injections in the three experimental groups. **A)** Jaw movements and cycle durations. **B)** Muscle activities.

	Jaw movements (μm)				
A	GAPE	L-CL excursion	A-P divergence	Lateral excursion	Ant dispersion
Control group					
before injection	1726 ± 230	943.7 ± 295.2	637 ± 141	1500 ± 332	146 ± 30
after injection	1694 ± 355	1046.4 ± 190.9	715 ± 109	1612 ± 183	155 ± 63
Significance	NS	NS	NS	NS	NS
Mas-hypofunction group					
before injection	2207 ± 340	1144.5 ± 226.7	798 ± 159	1463 ± 306	104 ± 34
after injection	1955 ± 384	1136.5 ± 344.1	833 ± 142	1837 ± 602	233 ± 78
Significance	*	NS	NS	NS	*
Temp-hypofunction group					
before injection	1938 ± 337	1086.7 ± 168.6	773 ± 178	1485 ± 276	112 ± 40
after injection	1785 ± 359	626.8 ± 219.1	507 ± 216	1128 ± 284	109 ± 24
Significance	NS	*	*	*	NS

	Cycle duration (ms)				
A	TC	OP	CL	E-CL	L-CL
Control group					
before injection	253.7 ± 29.4	140.7 ± 25.8	112.9 ± 10.5	60.2 ± 85.9	52.7 ± 15.1
after injection	267.0 ± 83.4	151.4 ± 63.4	115.6 ± 22.2	59.7 ± 12.9	55.9 ± 12.4
Significance	NS	NS	NS	NS	NS
Mas-hypofunction group					
before injection	240.6 ± 27.8	134.6 ± 19.7	106.0 ± 15.9	62.8 ± 9.0	43.1 ± 13.8
after injection	275.9 ± 27.8	152.6 ± 30.3	123.3 ± 13.0	65.6 ± 10.9	56.1 ± 13.0
Significance	*	NS	*	NS	*
Temp-hypofunction group					
before injection	227.9 ± 31.5	124.3 ± 24.1	103.7 ± 15.3	56.0 ± 10.6	47.7 ± 10.1
after injection	229.1 ± 43.5	122.5 ± 23.6	106.6 ± 25.0	70.5 ± 15.6	36.1 ± 12.9
Significance	NS	NS	NS	*	NS

	EMG duration (ms)		EMG area (AD per units)	
B	MAS	TEMP	MAS	TEMP
Control group				
before injection	60.7 ± 8.8	79.8 ± 8.2	0.0088 ± 0.0040	0.0069 ± 0.0025
after injection	63.7 ± 12.1	83.3 ± 18.4	0.0099 ± 0.0019	0.0072 ± 0.0031
Significance	NS	NS	NS	NS
Mas-hypofunction group				
before injection	58.0 ± 8.4	88.3 ± 14.7	0.0079 ± 0.0022	0.0061 ± 0.0022
after injection	43.1 ± 10.7	91.4 ± 15.7	0.0024 ± 0.0009	0.0056 ± 0.0019
Significance	*	NS	*	NS
Temp-hypofunction group				
before injection	56.7 ± 10.8	74.8 ± 10.7	0.0091 ± 0.0028	0.0057 ± 0.0016
after injection	52.5 ± 9.8	46.5 ± 26.1	0.0063 ± 0.0030	0.0021 ± 0.0050
Significance	NS	*	*	*

*P < 0.05; NS indicates not significant.

Values are presented as the standard deviation of the mean of each group. Individual differences were examined using a paired t-test.

Table 2. Comparison of jaw movements, cycle durations and muscle activity before and after BoNT/A injections in the three experimental groups. **A)** Jaw movements and cycle durations. **B)** Muscle activities.

*$P < 0.05$; NS indicates not significant.

Values are presented as the standard deviation of the mean of each group. Individual differences were examined using a paired *t*-test.

Figures

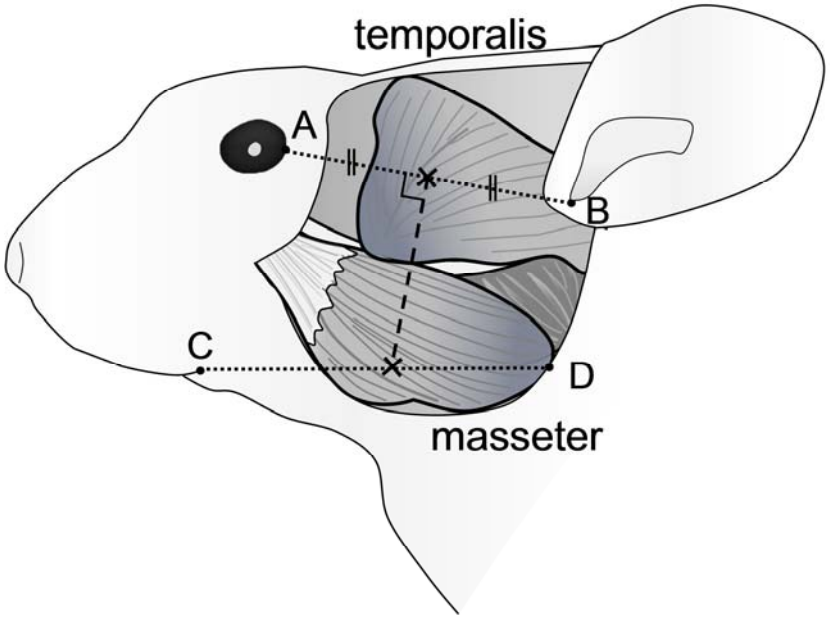
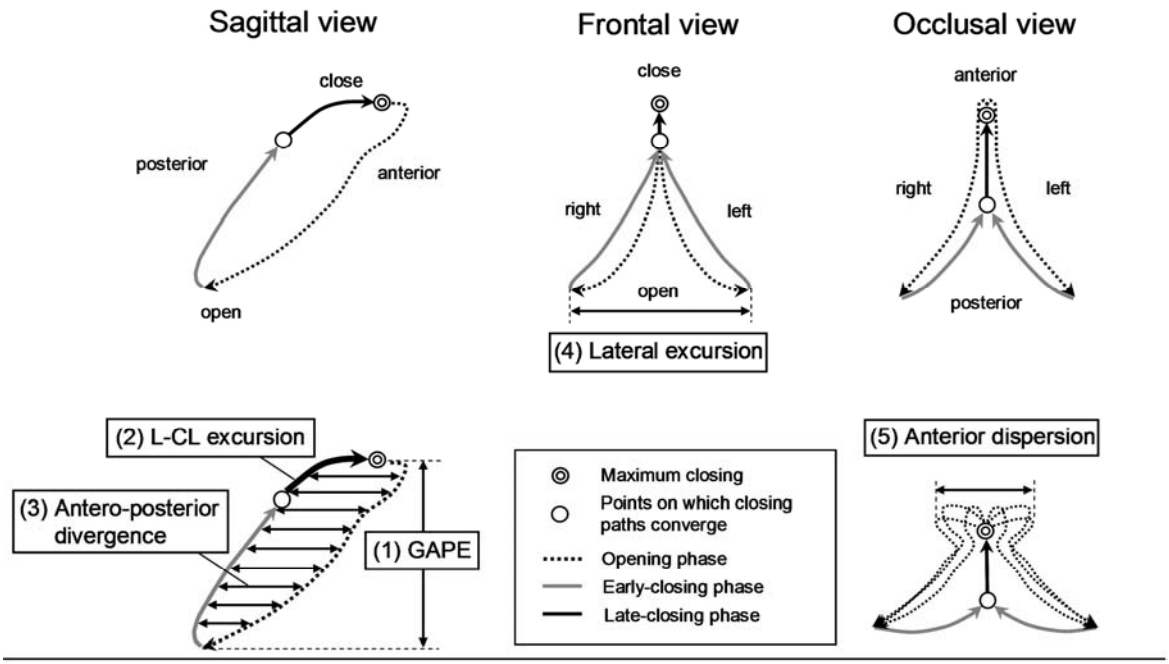


Fig. 1. Lateral schematic of the mouse masseter and temporalis muscles. Upper cross mark shows the injection site for the temporalis muscle. Lower cross mark shows the injection site for the masseter muscle. The injection site for the temporalis muscle is the midpoint of a line connecting the lateral canthus (A) to the auditory meatus (B). The injection site for the masseter muscle is the intersection of a perpendicular from the midpoint of line AB and a line connecting the outer oral commissure (C) to the mandibular angle (D).



Definitions of parameters used to analyze jaw movements

Parameters	Definition
(1) Gape size (GAPE)	Opening/closing excursion between maximum opening and maximum closing
(2) Late-closing phase excursion (L-CL excursion)	Distance along the curvature corresponding to the late-closing phase
(3) Antero-posterior divergence of opening and closing paths (Antero-posterior divergence)	The average of horizontal distance between two points in opening and closing path in which the jaw trajectory was divided vertically into ten equally spaced sections
(4) Lateral excursion	Distance of the two maximum opening points bilaterally
(5) Dispersion in the most anterior position in the lateral direction (Anterior dispersion)	Dispersion during anterior movement
(6) Total cycle duration (TC)	Time between one point of maximum jaw-opening and the next
(7) Opening phase duration (OP)	Time between maximum closing and the next maximum opening
(8) Closing phase duration (CL)	Time between maximum opening and the next maximum closing
(9) Early-closing phase duration (E-CL)	Time between maximum opening and the point at which closing paths converge
(10) Late-closing phase duration (L-CL)	Time between point at which closing paths converge and maximum closing

Fig. 2. Upper tracings: phase analysis of jaw movements. Dotted line, opening phase (OP); gray solid line, early-closing phase (E-CL); black solid line, late-closing phase (L-CL). Arrow indicates the direction of jaw movement. Open circle represents the point at which closing paths converge. Double circle represents maximum closure. Lower tracings: lines with arrows show the distances measured.

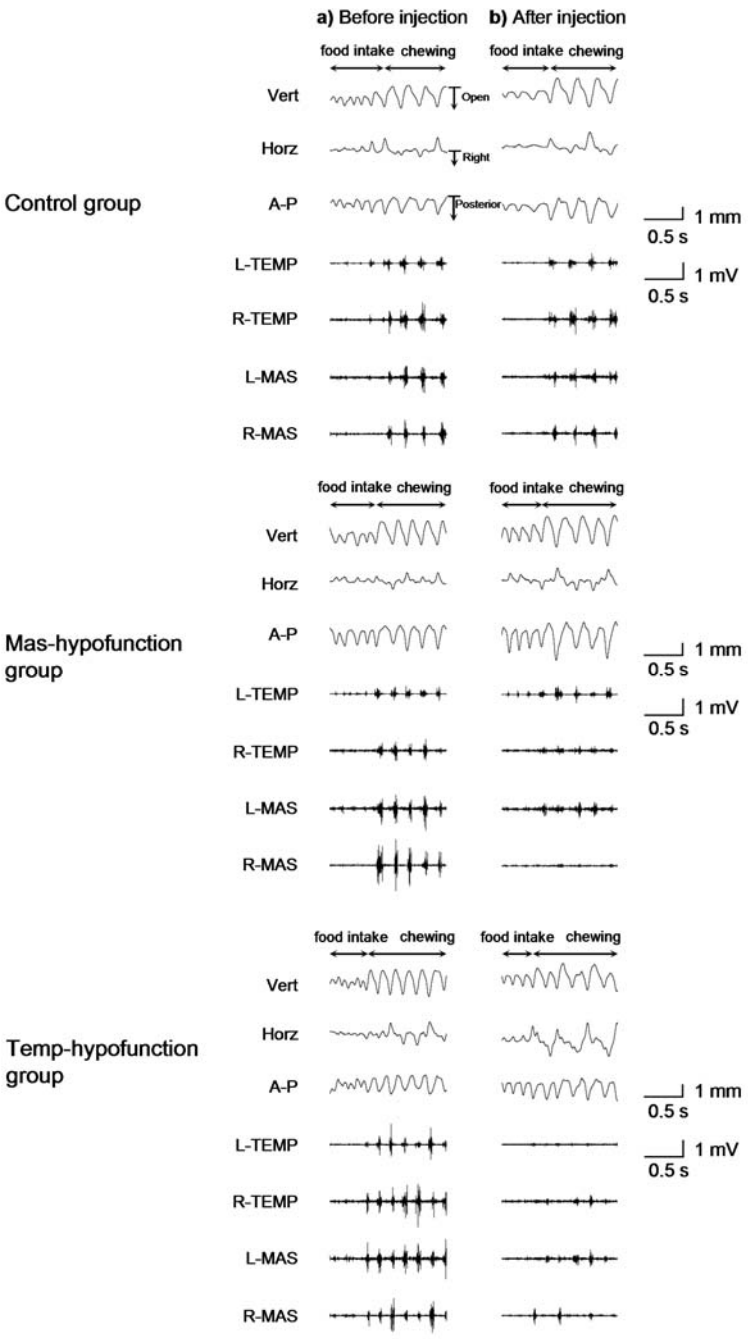


Fig. 3. Typical masticatory sequence. **a)** Before injection. **b)** After injection. The upper three traces illustrate jaw movements in the vertical (*Vert*), horizontal (*Horz*), and antero-posterior (*A-P*) directions. The lower three traces show electromyography (*EMG*) of the left temporalis (*L-TEMP*), right temporalis (*R-TEMP*), left masseter (*L-MAS*), and right masseter (*R-MAS*) muscles. The sequence was divided into food intake and chewing.

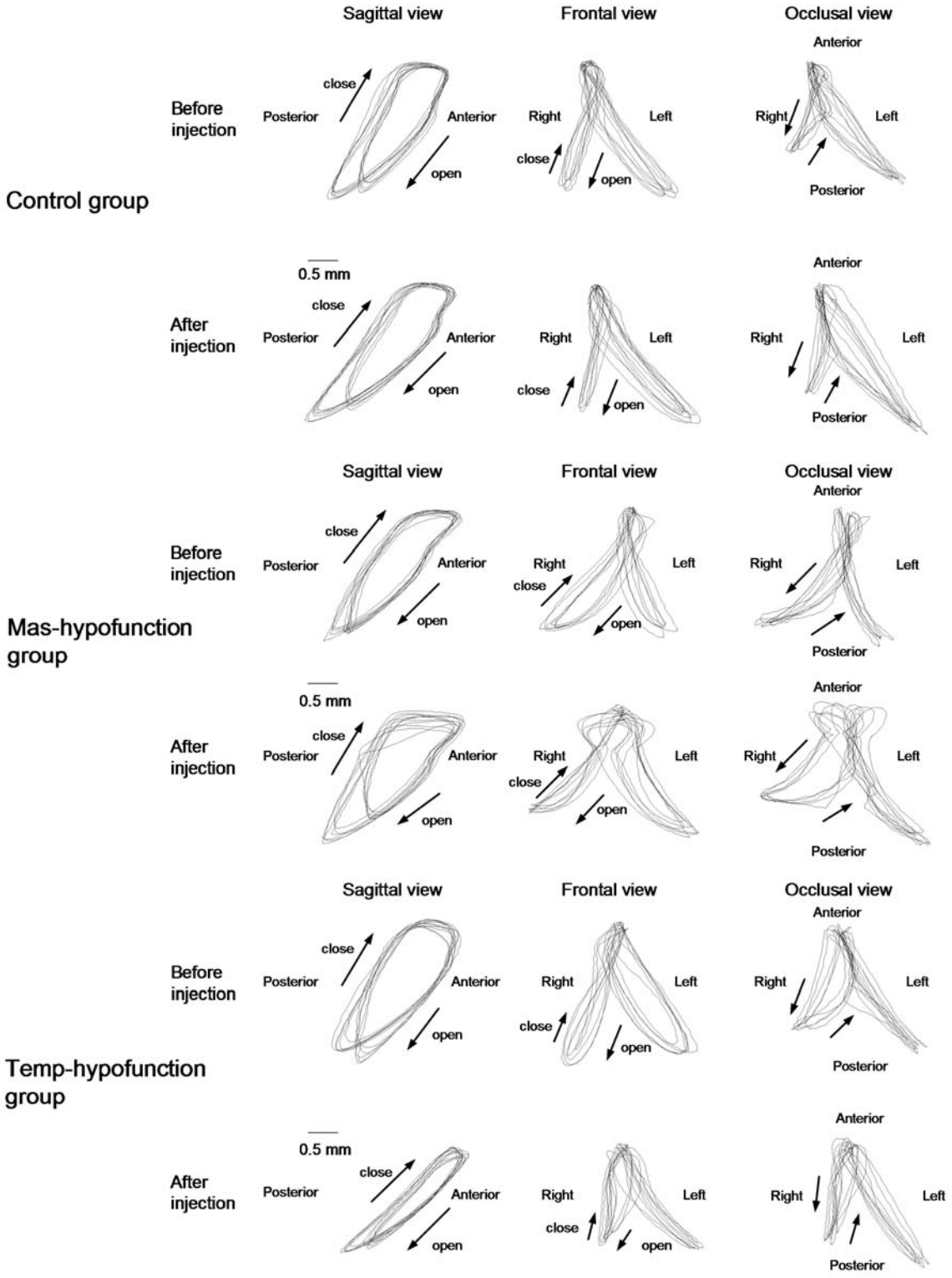


Fig. 4. Jaw-movement trajectories of the control (upper), masseter-hypofunction (middle), and temporalis-hypofunction (lower) groups in the sagittal (left), frontal (center), and occlusal (right) planes before and after injection. Tracings show 10 consecutive chewing cycles superimposed.