# Effects of Unilateral Nasal Obstruction on the Development of the Cortical Masticatory Area in Growing Rats

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# Abstract

The cortical masticatory area (CMA) regulates masticatory movements and controls rhythmic jaw movements. However, information regarding the effect of respiratory disturbances on the functional development of the CMA remains limited. This study aimed to examine the effect of chronic unilateral nasal obstruction (UNO) on the motor representation of the anterior digastric (AD) muscle during the growth period. Forty-eight 8-day-old male Wistar albino rats were randomized into control (CONT, n=24) and UNO (n=24) groups. Both groups underwent intracortical microstimulation (ICMS) at ages 5, 7, and 9 weeks. Arterial oxygen saturation ($SpO_2$) and the number of positive ICMS sites for the AD muscle were measured throughout the experiment. The $SpO_2$ values were significantly lower in the UNO group than in the CONT group at each age. Further, the number of positive ICMS sites for the AD muscle was significantly higher in the UNO group than in the CONT group at each age. Intragroup comparisons revealed that the number of positive ICMS sites increased with age. The onset latencies of the evoked AD potential significantly differed between the right and left sides. However, no differences were found between the CONT and UNO groups at any age. These findings suggest that UNO during development induces changes in the motor representation in the CMA.


# Introduction

Changes in breathing patterns due to nasal obstruction have been widely studied in recent years. Chronic nasal obstruction caused by adenotonsillar hypertrophy, hypertrophied turbinates, and allergic rhinitis are common in children and young adults (Stewart et al., 2010; Yadav et al., 2003). In particular, the number of children diagnosed with nasal breathing disorders during the growth period has been increasing, and chronic nasal breathing disorders have been reported to affect maxillofacial morphology and function (Abreu et al., 2008; Izu et al., 2010). Moreover, reductions in masticatory performance due to changing hypotonia of the lips, tongue, and buccinator muscles has been reported in children with nasal obstruction (Valera et al., 2003). Indeed, nasal septal curvature, allergic rhinitis, and nasal polyposis related to nasal breathing disorders have been associated with maxillary anterior protrusion, clockwise rotation of the lower jaw, and irregular occlusion of the dental arch (Harari et al., 2010).

Chronic nasal obstruction is known to reduce arterial oxygen saturation ($SpO_2$) and lung weight. Changes in hormonal behavior have also been reported in rat models of nasal obstruction (Padzys et al., 2011). Unilateral nasal obstruction (UNO) in a rat study induced changes in masticatory muscle activity, including reduced growth of the masseter muscle and anterior digastric (AD) muscles. As a result, the fatigue-resistant myosin heavy chain fiber type was increased (Gelhaye et al., 2006; Tang et al., 2019). In addition, it has been demonstrated that during the growth period, UNO reduces the corticofugal effects of intracortical microstimulation (ICMS) in the primary motor cortex. The primary motor cortex is also necessary for fine-tuning motor behaviors; encoding the muscles of the mouth, tongue, and jaw; and controlling orofacial motor functions (Abe et al., 2017). UNO influences oral function by delaying the conduction velocity and thereby delaying the jaw-opening reflex (Funaki et al., 2014). Additionally, early UNO may increase the contraction force of the tongue-protruding muscles and prolong the duration of

muscle contraction (Koecklin et al., 2015). Nasal breathing is considered to appropriately support the harmonious growth and development of the maxillofacial structure and interaction with orofacial functions such as chewing and swallowing (McNamara, 1981). Indeed, mammals, including humans, typically breathe through their noses. Several morphological changes and a reduction in masticatory function are induced by chronic nasal obstruction during adolescent development (Onozuka et al., 1999; Yamada et al., 1997; Tsubamoto-Sano et al., 2019). Furthermore, chronic nasal obstruction affects the area of the central nervous system that is responsible for memory and learning functions (Sakaguchi, 1999). Previous clinical studies have also reported that chewing time increases when nasal congestion occurs (Huang et al., 1989). Consequently, there is a close relationship between respiratory dynamics and the cortical masticatory area (CMA).

A research group at the University of Toronto conducted detailed electrophysiological studies on the cerebral cortices of monkeys (Narita et al., 1999; Hamdy et al., 1998). Further, following studies using a non-invasive brain activity measurement method in humans (Hamdy et al., 1998; Martin and Chao, 2001; Momose et al., 1997; Onozuka et al., 2002; Ferla et al., 2008), the role of the higher brain in the control of masticatory and swallowing movements is gradually becoming clear. The CMA is a higher brain area involved in the control of masticatory movements, including the major components of chewing behavior and muscular components in the oral and facial regions. Some studies have elucidated the effects of nasal obstruction on masticatory function, such as inhibition of masseter muscle activity and increased suprahyoid muscle activity (Ebner, 2005). Although alterations in craniofacial morphology and function during nasal obstruction have been thoroughly investigated, little is known about their effects on the central nervous system. Therefore, the purpose of this study was to elucidate the changes in the CMA caused by chronic UNO during the growth period.

# Materials And Methods

We performed ICMS to detect cortical representation in the CMA. The ICMS-induced electromyogram (EMG) was used to determine whether changes occurred in the motor representations of the masticatory muscles in growing rats following UNO. This is the first study to document the effects of UNO during growth on motor expression in the CMA. The rat CMA can be divided into the A-area and P-area, which are located in different places. The A-area is located in the primary motor cortex and the P-area is located on the ventral side of the insular cortex (Murray et al., 2001; Sessle, 2009; Sessle et al., 2007; Yao et al., 2002; Gogolla, 2017). The insular cortex receives information from outside the body (auditory, somatosensory, olfactory, gustatory, and visual information) (Maeda et al., 2014). This study was conducted in order to clarify the differences in the effects of UNO on the A-area and P-area. The null hypothesis was that chronic UNO during growth does not alter the expression of motor maps within the CMA and does not affect the maturation of the CMA.

All experiments described herein were approved by the Institutional Animal Care and Use Committee (No. A2019156 and No. A2020031) and performed in accordance with the Animal Care Standards of Tokyo Medical and Dental University.

# Nasal obstruction procedures

Forty-eight 8-day-old male Wistar albino rats were randomly divided into control (CONT, n = 24) and UNO (n = 24) groups. At 8 days of age, all rat pups were first anesthetized by hypothermia by placing them inside a chamber with an internal temperature of -18°C for 10 min. The tissues surrounding the external nostril were burned by the placement of a surgical cauterizing instrument (1 mm in diameter) on the right nostril, thus occluding the nostril without mechanical or chemical damage to the olfactory mucosa. To prevent infection, the nostrils were coated with 3% chlortetracycline (tetracycline hydrochloride paste 3%; Showa, Tokyo, Japan) after cauterization. The pups were then warmed (37°C) for 30 min and then returned to their mothers. In the CONT group, the cauterizing instrument was placed 1–2 mm above the right nostril, and the nostril was not sealed.

Body weights were measured at the time of the experiment. $SpO_2$ was recorded using a pulse oximeter (MouseOx Plus; STARR Life Sciences, Oakmont, PA, USA) in awake (non-anesthetized) rats using collar clip sensors. The sensors were placed dorsolaterally with the tips situated near the carotid arteries. $SpO_2$ signals were sampled at 1 Hz. The $SpO_2$ values were then averaged for 40–50 s of data.

# ICMS and EMG recordings

ICMS mapping was carried out when the rats were 5, 7, and 9 weeks of age in the CONT and UNO groups (n = 8 per group per time point). A cannula was inserted into the femoral vein, and ketamine HCl (100 mg/kg) (Ketalor: Daiichi Sankyo Propharma, Tokyo, Japan) was administered via injection initially for the craniotomy and EMG electrode insertion. Supplementary doses were injected whenever necessary to maintain a constant level of anesthesia throughout the ICMS mapping procedure, as indicated by vibrissa whisking, pinch-withdrawal, and eyelid reflexes. Ketamine anesthesia maintains muscle tone and is therefore well-suited for motor mapping studies (Abe et al., 2017). Throughout the study, rats were maintained under a constant level of anesthesia, as indicated by vibrissa whisking, pinch-withdrawal, and eyelid reflexes with intravenous infusion of ketamine-HCl. Furthermore, local anesthetic (lidocaine hydrochloride 2%) was injected into the subcutaneous space below the planned surgical area. Body temperature was maintained at a physiological level of 37–38°C using an electric blanket. Bipolar EMG electrodes (40-gauge, single-stranded, and Teflon-insulated stainless-steel wires) were used to record EMG activity from the left and right AD (LAD and RAD, respectively) muscles. The rats were then placed in a stereotaxic apparatus (models SN-2 and SM-15 M; Narishige Scientific Instruments, Tokyo, Japan) in the prone position. In the present study, craniotomies were performed over the left cortical hemispheres. The dura mater was kept intact and covered with warm mineral oil (37°C).

A fine glass-insulated tungsten microelectrode (shaft diameter of 100 μm with an impedance of 1–3 M at 1 kHz; Unique Medical, Tokyo, Japan) was inserted transmurally into the exposed hemispheres in a standardized systematic series of microelectrode penetrations. Electrical stimulation is the minimum stimulus that can confirm masticatory movements. The stimulation intensity required for CMA stimulation to induce masticatory movements in rats has been shown to vary between 50 and 300 μA

(Sasamoto et al., 1990; Satoh et al., 2007; Tsujimura et al., 2016; Asanuma, 1989). In addition, penetrations guided by a micro-drive had a horizontal spatial resolution of 0.5 mm, which coincided with the estimated extent of ICMS current spread of < 0.5 mm at an intensity of 60 μA (Neafsey et al., 1986; Swanson, 2018). In the present study, to prevent damage to the cerebral cortex, stimulation was performed at 60 μA, which was the minimum stimulation at which jaw movements could be observed. For the stimulation sites, the range was stimulated until the ICMS-positive reaction could not be confirmed. Electrical stimulation (0.5 ms duration, 30 Hz, 60 μA) was applied to the left anterior part of the CMA (2–4 mm rostral, 2–4 mm lateral to Bregma, 2–4 mm ventral), while electrical stimulation (0.5 ms duration, 30 Hz, 60 μA) was applied to the left posterior part of the CMA (-2–2 mm rostral, 4.5–6.5 mm lateral to Bregma, 4–6 mm ventral) (Satoh et al., 2007).

If no jaw movements were evoked with each electrical stimulation, the site was recorded as negative. The level of anesthesia was also monitored by revisiting the positive response sites. At each anteroposterior (AP) plane, a series of microelectrode penetrations in the mediolateral (ML) plane was applied until no ICMS-evoked EMG activities could be detected. We used Bregma as a reference point, as the relationship between the skull and brain structures varies among rats. There were also individual differences in the actual anatomical positions of the ICMS sites. Thus, the results from rats in which the coronal sections coincided with AP of the Swanson Brain Maps atlas (Ridding et al., 2000) were included in the statistical analysis.

# Data analysis

EMG activity was amplified and digitized using a multichannel amplifier (MEG-6108; Nihon Kohden, Tokyo, Japan; 1,000 gain, 300, and 3 kHz for low- and high-pass filters, respectively) followed by full-wave rectification and integration. All data were analyzed offline on a computer with a CED 1401 interface and Spike2 software for Windows (version 5.21; Cambridge Electronic Design, Cambridge, UK).

An ICMS site was defined and counted as a “positive ICMS site” if at least three of five ICMS trains evoked an EMG response in a muscle with an onset latency of a maximum of 40 ms and a peak activity that exceeded the mean value in the initial 10 ms of the EMG response plus two standard deviations (SDs) positive. A cortical site at which 60 μA ICMS could not evoke an EMG response was defined as an “unresponsive site.” A site at which the ICMS simultaneously evoked EMG responses in more than one muscle was defined as an “overlapping site.”

# Center of the gravity of cortical tissue devoted to the recruitment of muscles

To identify shifts in muscle representations, the center of gravity of the cortical tissue devoted to the recruitment of muscles, (ML coordinates from the sagittal suture/superoinferior [SI] coordinate from the cortical surface/AP coordinates to the Bregma), was calculated for the AD muscles by considering the mean number of positive ICMS sites obtained at the ML, SI, and AP coordinates in the CMA. This provided the position of the motor maps weighted relative to the extent of the motor representation

(Ridding and Rothwell, 1997; Paxinos, 2014). The following equation was used: $X=\Sigma a_i X_i / \Sigma ai$, where $a_i$ is the number of positive ICMS sites at the cortical ML coordinate, Xi. The SI coordinate Yi, and the AP coordinate Zi were determined in a similar manner.

# Recording the gap size of jaw movement

For recording sessions, a wire (0.7 mm thick) attached to a maker was placed between the lower incisors attached to the dental resin. A digital high-speed HAS-UIM camera (DITECT, Corp., Tokyo, Japan) was set directly in front of the maker to detect jaw movements. During stimulation, the jaw movements were videotaped, and 2D motion analysis system software (Dipp-motion V, DITECT, Cop., Tokyo, Japan) was used to refine the maker position of the jaw movements. The jaw movements were then stored on a computer. The jaw movement parameters measured were gap sizes (vertical excursion between the maximum opening and first position of the mandible). The mean values of the data for each parameter were measured from ten chewing cycles.

# Histological analysis

The rats were deeply anesthetized after ICMS and EMG recordings at 5, 7, and 9 weeks of age. The rats were perfused with 100 mL of phosphate-buffered saline (PBS; pH 7.4) through the left cardiac ventricle, followed by 300 mL of 4% paraformaldehyde for fixation. Cross sections of the brain (50 μm in thickness) were stained with hematoxylin-eosin. Previously described cytoarchitectonic features of the rat frontal cortex, in which the granular cortex is characterized by a prominent granular layer IV of densely packed cells and the absence of an agranular layer, were used to delineate the boundaries between the granular and agranular cortex in the coronal sections (Ridding et al., 2000; Brown and Sherrington, 1912). Sites located outside of the cortical gray matter were excluded from the data analysis.

# Statistical analysis

All data are expressed as the mean ± standard deviation (SD). An unpaired t-test was used for statistical comparisons of the mean body weight and $SpO_2$ between the CONT and UNO groups. Data normality was examined using the Shapiro – Wilk test. The number of positive ICMS sites, onset latencies, center of gravity of cortical tissue, and gap size of jaw movements were compared between the CONT and UNO groups and among age groups using a two-factor multivariate ANOVA (factors: treatment group, age). Simple main effects analysis with Sidak adjustment was used for multiple comparisons. Statistical analysis was performed using SPSS Statistics for Windows, version 22.0 J (SPSS, Chicago, IL, USA), and the level of significance was established at $P < 0.05$.

## Results

# Body weight and $SpO_2$

The mean body weight in the CONT and UNO groups increased normally throughout the experimental period. There was no significant difference in the mean body weight between the CONT and UNO groups

at any given age (Table 1). The mean values of $SpO_2$ for the UNO group were significantly smaller than those of the CONT group at each age (Table 2).

Table 1
Changes in body weight

| | **Weight(g)** | | | | |
|---|---|---|---|---|---|
| | Control | | Nasal obstruction | | |
| Age(week) | Mean | SD | Mean | SD | P |
| 5 | 136.2 | 15.5 | 126.5 | 21.7 | NS |
| 7 | 213.4 | 19.1 | 219.8 | 13.4 | NS |
| 9 | 271.7 | 14.4 | 282.6 | 16.4 | NS |
| Abbreviations: *P*, probability; *SD*, standard deviation; *NS*, not significant | | | | | |

Table 2
Changes in arterial oxygen saturation

| | **$SpO_2$(%)** | | | | |
|---|---|---|---|---|---|
| | Control | | Nasal obstruction | | |
| Age(week) | Mean | SD | Mean | SD | P |
| 5 | 97.4 | 0.4 | 93.4 | 1.3 | * |
| 7 | 96.5 | 1.3 | 93.4 | 1.3 | * |
| 9 | 96.1 | 1.3 | 92.1 | 1.3 | * |
| *: $p < 0.05$. Abbreviations: *P*, probability; *SD*, standard deviation; *NS*, not significant | | | | | |

# Number of total positive ICMS sites in the A-area and P-area

The number of total positive ICMS sites in the CONT and UNO groups is shown in Figs. 1 and 2. In the A-area, intragroup comparison in the CONT group showed that the numbers of positive ICMS sites at 7 and 9 weeks of age were significantly larger than that at 5 weeks of age. The number of positive ICMS sites at 9 weeks of age was also significantly larger than that at 7 weeks of age. Intragroup comparison in the UNO group revealed that the numbers of positive ICMS sites were significantly larger at 7 and 9 weeks of age than at 5 weeks of age, and at 9 weeks of age than at 7 weeks of age. Further, the number of positive ICMS sites was significantly larger in the UNO group than in the CONT group at 5, 7, and 9 weeks of age. In the P-area, intragroup comparison in the CONT group indicated that the numbers of positive ICMS sites at 7 and 9 weeks of age were significantly larger than that at 5 weeks of age. The number of positive ICMS sites at 9 weeks of age was significantly larger than that at 7 weeks of age. Intragroup comparison

in the UNO group demonstrated that the numbers of positive ICMS sites was significantly larger at 7 and 9 weeks of age than at 5 weeks of age. In addition, the number of positive ICMS sites was significantly larger at 9 weeks of age than at 7 weeks of age. When the CONT group was compared to the UNO group, the number of positive ICMS sites was significantly larger in the UNO group than in the CONT group at 5, 7, and 9 weeks of age.

# Number of positive ICMS sites on the contralateral side in the A-area

The number of positive ICMS sites in the A-area is shown in Fig. 1. Intragroup comparison in the CONT group showed that the number of positive ICMS sites for the RAD muscle at 9 weeks of age was significantly larger than those at 5 and 7 weeks of age, but there was no significant difference between 5 and 7 weeks of age. Intragroup comparison in the UNO group revealed that the number of positive ICMS sites for the RAD muscle was significantly larger at 9 weeks of age than at 5 and 7 weeks of age, but there was no significant difference between 5 and 7 weeks of age. The number of positive ICMS sites for the RAD muscle was significantly larger in the UNO group than in the CONT group at 5 and 7 weeks of age.

# Number of positive ICMS sites on both sides in the A-area

The number of positive ICMS sites for the RAD and LAD muscles in the CONT and UNO groups is indicated in Fig. 1. Intragroup comparison in the CONT group showed that the numbers of positive ICMS sites for the RAD and LAD muscles at 7 and 9 weeks of age were significantly larger than that at 5 weeks, but there was no significant difference between 7 and 9 weeks of age. Intragroup comparison in the UNO group revealed that the numbers of positive ICMS sites for the RAD and LAD muscles at 7 and 9 weeks of age were significantly larger than that at 5 weeks. Further, the number of positive ICMS sites at 9 weeks of age was significantly larger than that at 7 weeks of age. When the CONT group was compared to the UNO group, there was no significant difference in the number of positive ICMS sites at 5, 7, and 9 weeks of age.

# Number of positive ICMS sites on the contralateral side in the P-area

The number of positive ICMS sites in the P-area is illustrated in Fig. 2. Intragroup comparison in the CONT group showed that the numbers of positive ICMS sites for the RAD and LAD muscles were significantly larger at 7 and 9 weeks of age than at 5 weeks of age, and the number of ICMS sites was significantly larger at 9 weeks of age than at 7 weeks of age. Intragroup comparison in the UNO group indicated that the numbers of positive ICMS sites were significantly larger at 7 and 9 weeks of age than at 5 weeks of age. Additionally, the number of positive ICMS sites was significantly larger at 9 weeks of age than at 7 weeks of age. When the CONT group was compared to the UNO group, the number of positive ICMS sites in the UNO group was significantly larger than that in the CONT group at 5, 7, and 9 weeks of age.

# Number of positive ICMS sites on both sides in the P-area

Intragroup comparison in the CONT group showed that there was no significant difference in the numbers of positive ICMS sites at 5, 7, and 9 weeks of age. Intragroup comparison in the UNO group revealed no significant difference in the numbers of positive ICMS sites at 5, 7, and 9 weeks of age. Moreover, when the CONT group was compared to the UNO group, there was no significant difference in the numbers of positive ICMS sites at 5, 7, and 9 weeks of age.

# Onset latency of ICMS-evoked EMG activities in the CMA

The onset latencies of the ICMS-evoked EMG activities in the CMA are presented in Fig. 3. The onset latencies of the evoked RAD and LAD EMG activities did not significantly differ between the two groups at any given age. However, the mean onset latencies were significantly shorter for the RAD than for the LAD at each age in both the CONT and UNO groups. The results were similar for the A-area and P-area.

# Center of the gravity of cortical tissue devoted to the recruitment of the AD muscle

The centers of gravity of cortical tissue devoted to the recruitment of the AD muscle, which reflects the mean three-dimensional center positions of the motor representations for the AD muscles, are shown in Fig. 4. The directions of the three axes are indicated in the rat brain (Fig. 4). We calculated the center of gravity for each A-area and P-area. The centers of gravity of cortical tissue devoted to the recruitment of the total muscles in the CONT and UNO groups in the A-area are shown in Fig. 4. Intragroup comparison in the CONT group revealed that the center of gravity shifted significantly to rostral from 5–9 weeks of age and laterally from 7–9 weeks of age. However, there was no significant difference in depth at any given age. Intragroup comparison in the UNO group indicated that the centers of gravity shifted significantly to rostral and lateral from 5–9 weeks of age. However, there was no significant difference in the depth at any given age. Compared to the UNO group, the centers of gravity in the CONT group shifted significantly laterally at any given age, but there was no significant difference in the rostral shift and depth between the CONT and UNO groups at any given age.

The centers of gravity of cortical tissue devoted to the recruitment of the total muscles in the CONT and UNO groups in the P-area are shown in Fig. 4. Intragroup comparison in the CONT group indicated that the center of gravity shifted significantly caudally from 5–9 weeks of age. Intragroup comparison in the UNO group revealed that the centers of gravity shifted significantly caudally and laterally and to greater depths from 5–9 weeks of age. Compared to the CONT group, the centers of gravity in the UNO group shifted significantly rostrally at 5 weeks of age and laterally at 7 and 9 weeks of age.

# Gap size of jaw movement

The gap size of the jaw movement is indicated in Fig. 5. The maximum opening volume was measured and compared when the CMA was continuously stimulated. In the A-area of the CONT group, the gap

sizes were 0.36, 0.50, and 0.72 mm at 5, 7, and 9 weeks, respectively. In the A-area of the UNO group, the gap sizes were 0.34, 0.46, and 0.68 mm at 5, 7, and 9 weeks, respectively. In the A-area, neither the CONT group nor the UNO group exhibited any change in the gap size of jaw movement with age.

In the P-area of the CONT group, the gap sizes were 0.32, 0.44, and 0.64 mm at 5, 7, and 9 weeks, respectively. In the P-area of the UNO, the gap sizes were 0.26 mm, 0.41, and 0.60 mm at 5, 7, and 9 weeks, respectively. There was no significant difference in the gap size of jaw movements between the CONT and UNO groups.

## Discussion

This study is the first to demonstrate that UNO during development can modify the expression of the motor maps within the CMA and affect the maturation of the CMA, which manifests as changes in the ICMS-defined motor representations of the AD muscles.

# Reliability of the ICMS-defined motor maps

In the present study, microelectrodes were used to stimulate the CMA. The ICMS procedure was performed by inserting microelectrodes into the brain, and the contraction of neuron-controlled muscles activated during stimulation was observed. Large sources of variability in the movements evoked by ICMS (e.g., variability among individuals, with different levels of anesthesia, different types of anesthetic agents, different pulse train durations, as a function of previous stimulation, etc.) is common, as previously discussed (Swanson, 2018; Brown and Sherrington, 1912; Hall and Lindholm, 1974; Lashley, 1923; Nudo et al., 1990; Penfield and Boldrey, 1937; Stoney et al., 1968; Nudo et al., 1992). In this study, we attempted to control several sources of variation caused by the ICMS technique. The large number of penetration sites provided a built-in reliability estimate of the local topography. ICMS mapping was conducted during stable periods of anesthesia. In addition, mapping was temporarily suspended during occasional periods of excessive muscle tone in the AD muscles, or during occasional periods of deep narcosis marked by movement threshold elevation (Fujiwara, 1990). Mapping was conducted by the procedure from the removal of the dura, specifically, penetration, which avoided the surface vasculature and minimized local ischemia caused by ruptured or temporarily occluded blood vessels.

*Growth-related changes in the number of positive ICMS sites within the A-area and P-area of the CMA*

In the present study, the total number of positive ICMS sites in both the A-area and P-area increased until 9 weeks of age in both the CONT and UNO groups. Rats begin masticatory activity at 3 weeks of age (Kawamura, 1989) and it has been reported that mastication after weaning results in abundant afferent stimulation and promotes brain development (Hou et al., 2017). Neuroplasticity is related to learning, and brain plasticity is indispensable for the establishment of learning and memory. Masticatory learning occurred during the growth period, and may explain the increase in the number of positive ICMS sites. Similarly, the total number of positive ICMS sites in the primary motor cortex increases with growth, and reportedly peaks at 9 weeks of age (Abe et al., 2017). Small amounts of chondroitin sulfate have been

shown to have a growth-promoting effect on the pediatric brain (Levrini et al., 2015). Thus, it is possible that chondroitin sulfate was associated with brain development in the current study.

*Effects of UNO on the number of positive ICMS sites within the A-area and P area of the CMA*

In the present study, the $SpO_2$ values of the UNO group were significantly smaller than those of the CONT group at each age. UNO in growing rats changes the properties of the respiratory and orofacial muscles (Padzys et al., 2011; Funaki et al., 2014; Koecklin et al., 2015; Zicari et al., 2014). UNO is associated with an initial decrease in lung growth, followed by recovery by 90 days of age (Padzys et al., 2011). The AD muscles of rats undergoing nasal obstructive treatment contain more myosin heavy chain (MHC)-II-X. As a result, the muscle generates a greater maximum specific force, faster shorting velocity, and lower resistance to fatigue (Padzys et al., 2011; Tang et al., 2019). UNO may lead to respiratory muscle adaptation and produce motor modifications associated with alterations in specific EMG activity of the jaw-opening muscles.

In the current study, a significant increase in the number of positive ICMS sites was noted in the UNO group. Nasal breathing disorders in humans cause posterior positioning of the mandible (Rosenzweig et al., 1962). In rats, when the $SpO_2$ decreases due to nasal breathing disorders, it has been observed that the mandible undergoes clockwise rotation to compensate for the decreased oxygen and is pulled backward and downward (Zicari et al., 2014). As a result of UNO, it has been reported that the MHC of the AD muscles is increased and muscle development is promoted (Padzys et al., 2011).

Animals were greatly affected by environmental factors if they are exposed to the same genetic and nutritional factors (Aghajanian and Bloom, 1967). Prior studies suggested that rats between 4 and 8 weeks of age are the most affected by environmental factors, which induce a period of systematic and functional abrupt changes in the cerebrum (Altman, 1969; Diamond et al., 1975; Kurihara, 2016). The nervous system exhibits plasticity to avoid dysfunction or compensate for dysfunction. Clinical evidence shows that neuroplasticity, which occurs when the brain is injured, is stronger in early childhood (Gordon and Stryker, 1996). In addition, brain development has been demonstrated to facilitate functional recovery (Gordon and Stryker, 1996), and yields a strong shift in cortical responsiveness toward the non-deprived eye, concomitant with the remodeling of dendritic spines and axons during the critical period (Kikuta et al., 2008). In the present study, UNO was performed at 5, 7, and 9 weeks of age, which correspond to the period of systematic and functional abrupt changes in the cerebrum. Thus, the chronic contraction of the AD muscles may have been due to the effect of UNO, which generated an increase in afferent stimulation from the periphery, resulting in neuroplastic changes and an increase in the number of positive ICMS sites.

*Difference between the A-area and P-area in the number of positive ICMS sites on the contralateral side*

In the present study, the total number of positive ICMS sites exhibited a similar increase in both the A-area and P-area. However, the number of positive ICMS sites of the RAD muscle on the contralateral side of the brain stimulation differed between the A-area and P-area. Until 7 weeks of age, the number of positive

ICMS sites of the RAD muscle underwent a similar increase in the A-area and P-area. However, only the P-area showed an increase in positive ICMS sites at 9 weeks of age. Thus, it is assumed that the A-area at 9 weeks of age is not affected by UNO.

The present study confirmed that the difference in the number of positive ICMS sites in the A-area is located in the primary motor cortex, whereas the P-area is located in the ventral part of the insular cortex (Sasamoto et al., 1990). The insular cortex controls the senses of smell and taste.

The sense of smell was greatly affected by UNO. The neurons in the anterior olfactory nucleus of the rat olfactory cortex are ordinarily dominated by ipsilateral inputs, and bi-nasal neurons in the anterior olfactory nucleus respond to ipsilateral and contralateral nasal inputs with nearly equivalent odorant category selectivity (Coppola, 2012). On the occluded side, the airflow is dramatically reduced, particularly rostral to the nasopharyngeal canal. The open side is forced to carry a larger-than-normal volume of air (Ren et al., 2019). In addition, UNO abrogates alternating cycles of breathing, forcing constant duty on the open side. Not surprisingly, this leads to detectable histological and physiological changes in the contralateral mucosa (Ren et al., 2019). Previous studies have reported that UNO also affects taste, particularly sweet taste perception via the reduced expression of taste-related molecules in the taste cells of rat circumvallate papillae (Tasaka et al., 2012).

In the present study, the insular cortex area changed with the increase in airflow; correspondingly, the number of positive ICMS sites on the contralateral side in the P-area increased. In the present study, it is suggested that the insular cortex is significantly affected by changes in the sense of smell and taste by UNO. The difference in brain areas led to differences in the number of positive ICMS sites on the contralateral side in the A-area and P-area. The changes in the gustatory cortex induced by UNO are unclear and will need to be investigated in the future.

# The change in the center of gravity of the CMA with growth

In the present study, the depth of the center of gravity showed almost no change, but the center of gravity significantly shifted in the rostral direction from 5 to 9 weeks of age and in the lateral direction from 7 to 9 weeks of age in the A-area. In the P-area, the depth of the center of gravity again showed almost no change, but the center of gravity significantly shifted in the caudal direction from 5 to 9 weeks of age and in the lateral direction from 7 to 9 weeks of age.

In the primary motor cortex mapping study, it was revealed that the change in neuroplasticity due to UNO was a lateral change in the center of gravity of the positive ICMS site (Abe et al., 2017). This is indicative of reorganization of motor expression in the primary motor cortex (Yadav et al., 2003; Abe et al., 2017). A previous study revealed the mechanism by which neurons determine the shape and direction of their dendritic trees in the cerebral cortices of mice (Nakazawa et al., 2018); it was found that intense competition for the survival of dendritic trees occurs when input is received only from a specific direction. As a result, dendritic trees that extend in the direction of the input survive and grow larger (Avivi-Arber et al., 2010a). This determines the direction of nerve development. Given the above, it can be concluded that

the CMA was subjected to input from a specific direction. The neuroplastic changes in the CMA may have been affected by changes in other cerebral cortical areas. In this study, the CMA was also affected as UNO caused a lateral change in the center of gravity of the primary motor cortex.

When the brain grows, the directions of expansion are different because of the difference in the input direction to the dendritic trees between the A-area and P-area. It is believed that the development of the brain is directional. Hence, it is thought that among the changes in neuroplasticity, neuroplasticity with lateral and anterior-posterior selectivity was induced.

# The effect of UNO on latency

There was no significant change in the onset latency between the UNO and CONT groups at any age. This indicates that UNO had no significant effect on the development of the fastest corticofugal projections to the CMA. Few studies have directly investigated the effect of chronic nasal obstruction on myelination. However, many previous studies have shown that orofacial environmental changes do not affect the onset latencies of ICMS-evoked EMG activity (Kato et al., 2012; Asanuma et al., 1976). When the onset latency shortens, subcortical synaptic efficacy increases (Butovas and Shwarz, 2003; Avivi-Arber et al., 2010b).

In the present study, the lack of significant changes in onset latencies supports the possibility that at least some of the observed CMA changes were a result of cortical neuroplastic changes rather than subcortical changes in the excitability of corticofugal projections. A significant contralateral dominance was reflected in the difference in the latency of ICMS-stimulated LAD and RAD muscles in the CONT and UNO groups. This may be attributed to the differences in the pathways to the RAD and LAD muscles. These findings are consistent with those of previous studies in rat RAD and LAD muscles (Yao et al., 2002; Kato et al., 2012; Kamen and Gabriel, 2010).

# The changes in gap size of jaw movement due to UNO

In this study, there was no significant difference in the gap size of jaw movement between the CONT and UNO groups at 5, 7, and 9 weeks of age. The peak-to-peak amplitude measurement method evaluates the active contractile force determined by the number of muscle fibers and the proportional number of motor units activated by electrical stimulation (Keenan et al., 2005; Das and Gilbert, 1995).

It is reported that partial retinal destruction temporarily stops responding to visual stimuli, but after a while, the input from the horizontal axons of the pyramidal cells in the surrounding normal site is strengthened and becomes visually responsive (Juliano et al., 2009; Hiraba and Sato, 2004). In a study of the CMA in cats, it was reported that even if the entire CMA was destroyed, the function was restored after 1 month (Hiraba and Sato, 2004). In the current study, it is speculated that the number of ICMS-positive reaction sites increased in order to recover function from nasal respiratory disturbances in the UNO group. It is assumed that the degree of opening did not change between the CONT and UNO groups because of neuroplasticity and functional recovery in the UNO group. Thus, the increase in the number of positive ICMS sites may have occurred as a central response to compensate for the dysfunction.

# Conclusions

UNO in rats during growth periods induced changes in the $SpO_2$ and altered the number of positive ICMS sites within the CMA. These findings suggest that UNO occurring during developmental periods may affect not only respiratory function, but also orofacial function in rats. Therefore, the development of respiratory dysfunction should be monitored and corrected as soon as possible to avoid any complications in physiological function.

# Declarations

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**Availability of data and material:** Not Applicable

**Code availability:** Not Applicable

**Ethics approval:** All experiments described herein were approved by the Institutional Animal Care and Use Committee (No. A2019156 and No. A2020031) and performed in accordance with the Animal Care Standards of Tokyo Medical and Dental University.

**Consent to participate:** Not Applicable

**Consent for publication:** Not Applicable

**Authors' contributions:** A.F., C.K., S.K., H.O., Y.A., T.O., P.T.A., H.I., R.L., and T.O. conceived and designed research; A.F., C.K., and P.T.A. performed experiments; A.F., C.K., S.K., H.O., Y.A., T.O., P.T.A., H.I., R.L., and T.O. analyzed data; A.F., C.K., S.K., H.O., Y.A., T.O., P.T.A., H.I., R.L., and T.O. interpreted results of experiments; A.F., C.K., S.K., and T.O. prepared figures; A.F., C.K., and T.O. drafted manuscript; A.F., C.K., S.K., and T.O. edited and revised manuscript; A.F., C.K., S.K., H.O., Y.A., T.O., P.T.A., H.I., R.L., and T.O. approved final version of manuscript.

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# Figures

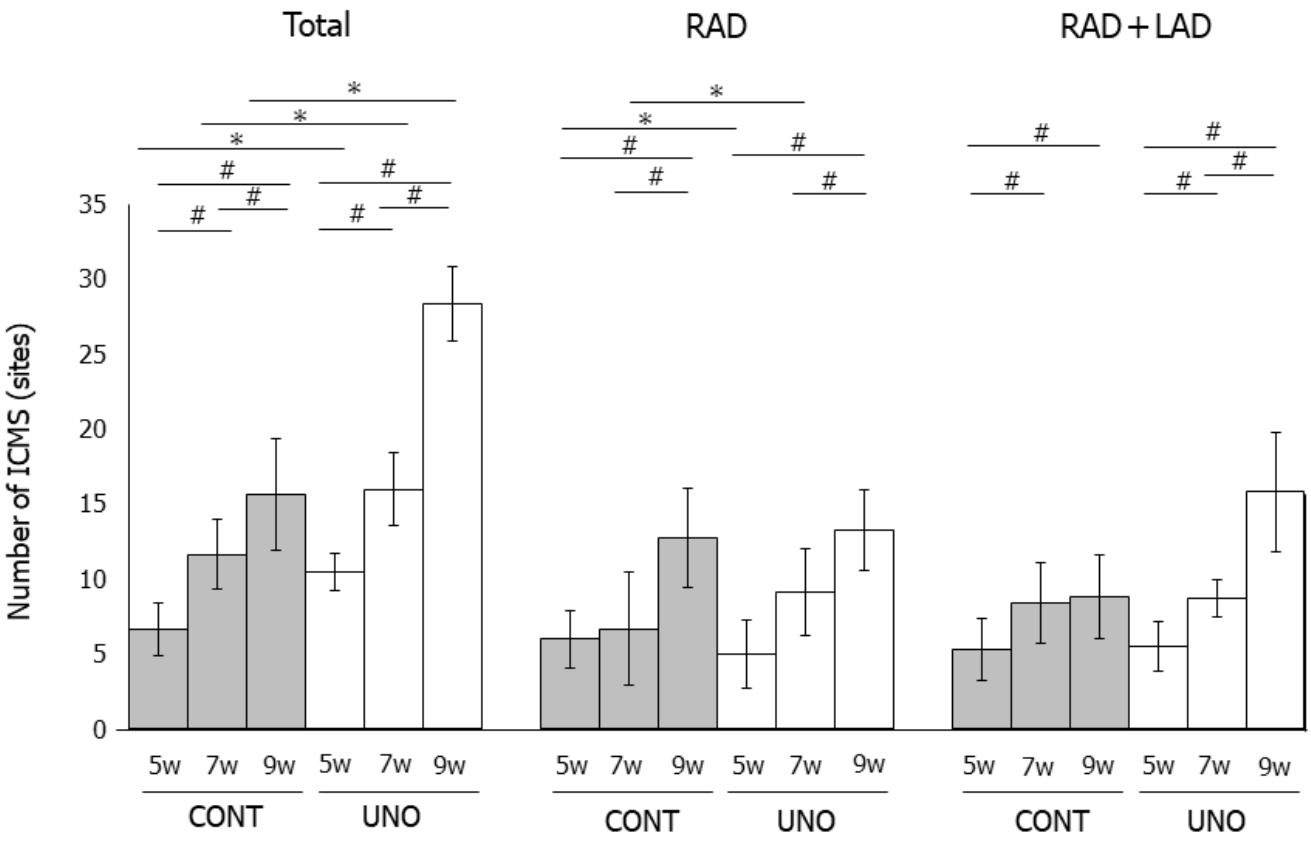



Fig. 1

## Figure 1

Number of the independent positive ICMS sites within the A-area of cortical masticatory area. Error bars indicate means ± standard error (SE). *: P<0.05, significant differences between the CONT and UNO groups at the same ages. #: P<0.05, significant differences between the different ages in each of CONT and UNO groups. Abbreviations: ICMS, intracortical microstimulation; RAD, right anterior digastric muscle; LAD, left anterior digastric muscle; CONT, control; UNO, unilateral nasal obstruction.

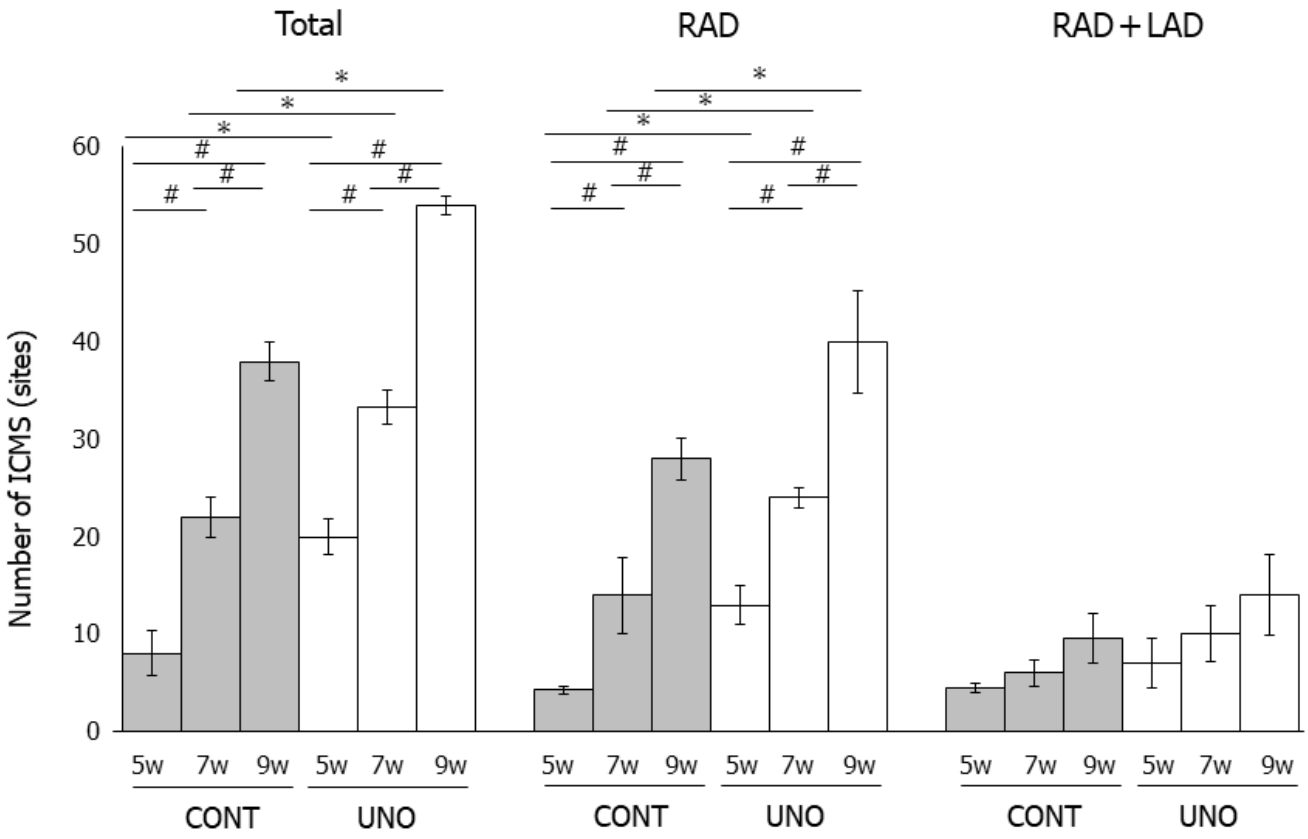



Fig. 2

## Figure 2

Number of the independent positive ICMS sites within the P-area of cortical masticatory area. Error bars indicate means ± standard error (SE). *: P<0.05, significant differences between the CONT and UNO groups at the same ages. #: P<0.05, significant differences between the different ages in each of CONT and UNO groups. Abbreviations: ICMS, intracortical microstimulation; RAD, right anterior digastric muscle; LAD, left anterior digastric muscle; CONT, control; UNO, unilateral nasal obstruction.

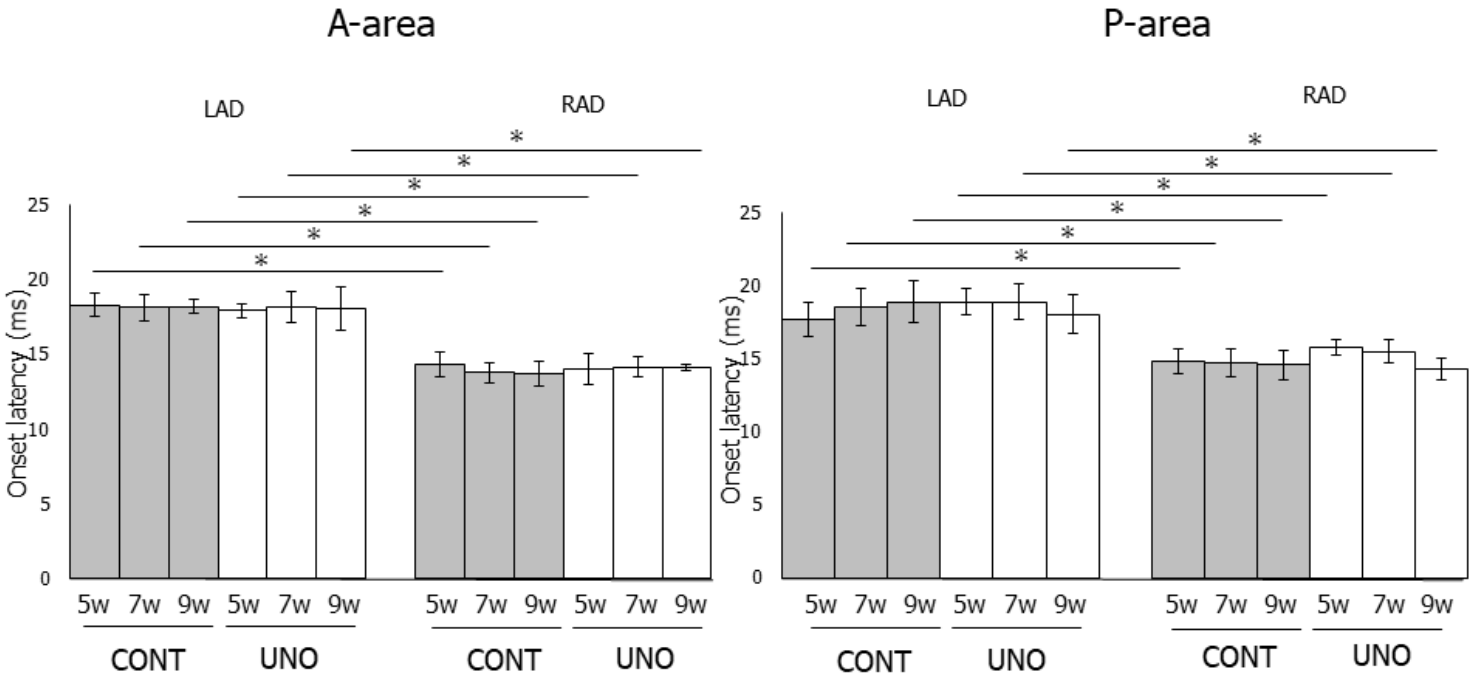
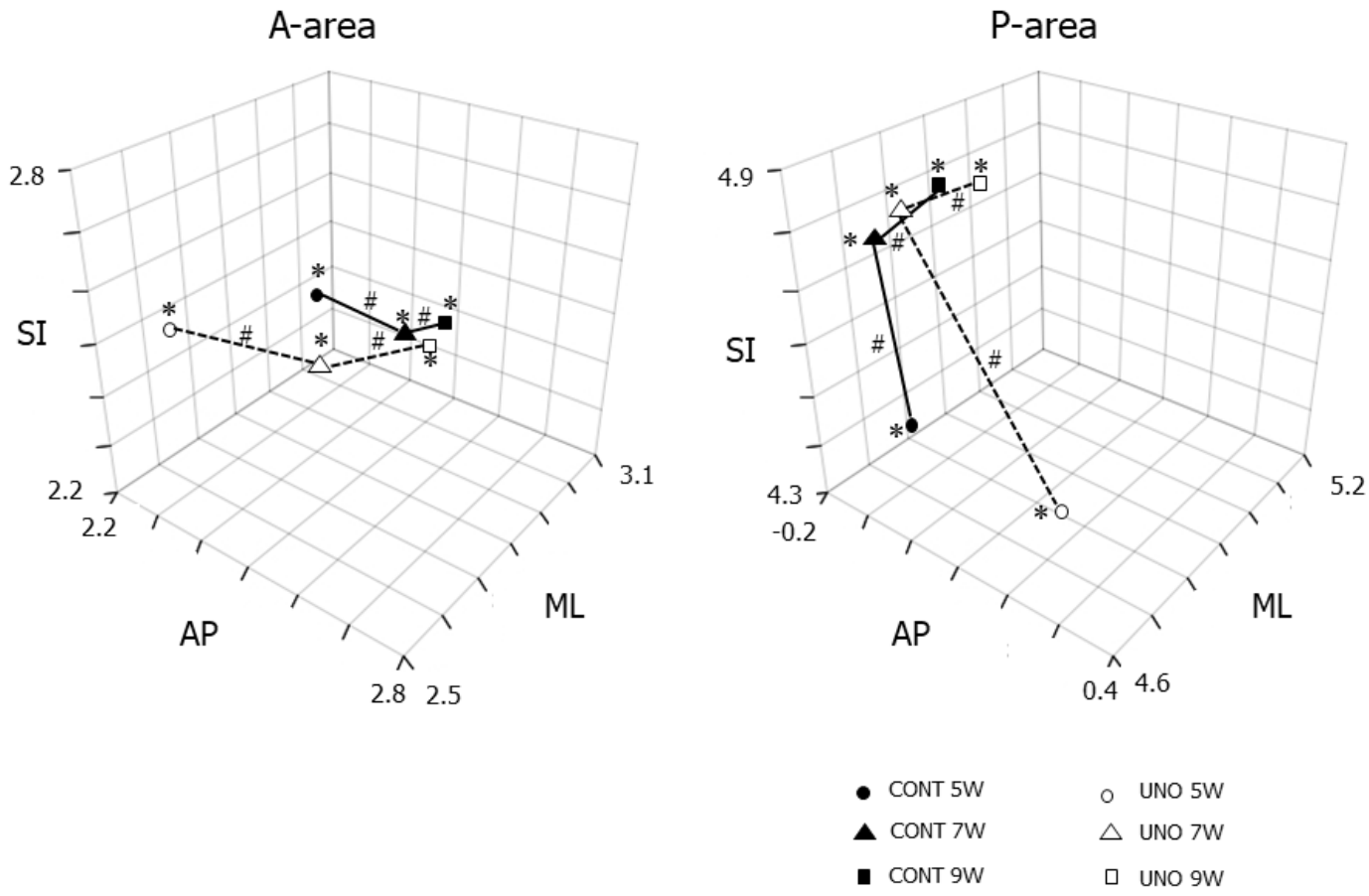



Fig. 3

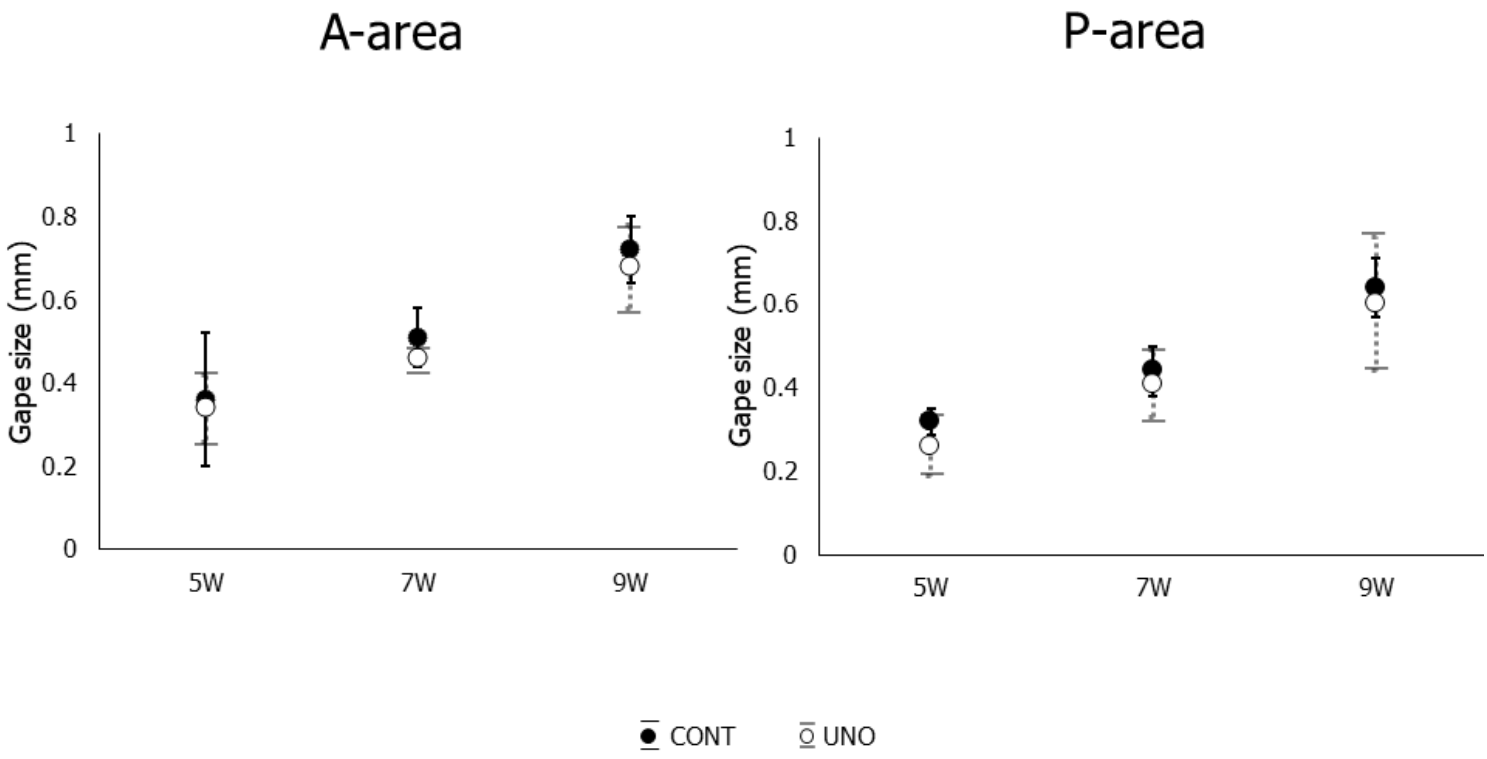
## Figure 3

Onset latencies of EMG activities in RAD, and LAD muscles evoked by ICMS. Error bars indicate mean ±standard error (SE). *: P<0.05, significant differences between the RAD and LAD muscles at the same age. Abbreviations: ICMS, intracortical microstimuration ; RAD, right anterior digastric; LAD, left anterior digastric ; CONT, control; UNO, unilateral nasal obstruction.




## Figure 4

Gravity of cortical tissue. A: the directions of the three axes are indicated on the rat. AP, anteroposterior; ML, mediolateral; SI, superoinferior. Depicted is the center of the gravity of cortical tissue devoted to recruitment of one muscle for left anterior digastric (LAD) and right anterior digastric (RAD) in 3-dimensional space. Numbers depict the distance from Bregma (mm). *P<0.05, significant intergroup differences at the same ages. #P<0.05, significant intragroup differences between 5 and 7 weeks of age, 7 and 9 weeks of age.




## Figure 5

Comparison of the path of maximum jaw opening between the control (CONT) and unilateral nasal obstruction (UNO) groups during maximum jaw opening. There is no significant differences between the CONT and UNO groups.