Skeletal effects of the alteration of masseter muscle function

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Aim: To investigate the effects of muscle denervation and the introduction of the β2-adrenoceptor agonist, formoterol, on the relationship between muscles and underlying skeletal growth.

Method: Thirty-one (4-week-old) male Sprague-Dawley rats were assigned to four groups: Surgical Sham; Denervated; Denervated + β2-agonist; and β2-agonist only. The Surgical Sham group had the left masseteric nerve exposed but not sectioned. Both of the denervated groups had the left masseteric nerve exposed and sectioned. The groups receiving the β2-agonist had formoterol directly injected into the left masseter muscle every three days for eight weeks. Sixteen angular and linear skeletal measurements were assessed in the overall craniofacial region and the mandible via standardised digital radiography in three views: lateral head, submento-vertex and right and left disarticulated hemi-mandibles.

Results: The findings indicated that, following surgical denervation of the masseter muscle, there were significant changes in the muscle and in the subsequent development of the underlying skeletal structures. The post-surgical changes were largely offset by the administration of a β2-agonist, formoterol, which attenuated muscle atrophy. However, the administration of the β2-agonist only, without surgical denervation, did not lead to changes in skeletal facial form.

Conclusions: Denervation atrophy of the masseter muscle results in statistically significant changes in the development of the underlying skeleton. The changes, however, are localised to areas of muscle attachment. The administration of the β2-agonist, formoterol, despite its effect on muscle anabolism, does not have a significant effect on underlying skeletal growth.

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Introduction

Much has been written about the relationship between masticatory muscles and the growth and development of the underlying craniofacial skeletal structures. The mandibular musculature’s influence on craniofacial development is yet to be determined conclusively, although it is generally accepted that craniofacial shape is under the influence of both genetic and environmental factors. The functional matrix theory describes the influence of soft tissues on facial form. It theorises that facial skeletal growth occurs in response to functional needs.

Previous research has used animal experimental models to explore the relationship between craniofacial morphology and muscle function. The studies have included changing the consistency of diet, physically removing the masseter, altering masticatory muscle function during growth, the removal of a sensory or motor neural branch, pharmacological denervation, and altering the expression of muscle-specific genes. Decreased muscle function and muscle denervation results in structural and functional changes to skeletal muscle including atrophy and a decrease in force-producing capacity. The examination of the musculoskeletal interaction in craniofacial growth had been limited in surgical and extirpation studies in animals until the introduction of non-invasive muscle imaging.
techniques, such as CT scanning, MRI, and ultrasonography. Skeletal changes have traditionally been observed with radiography, in which a standardised cephalostat is used to maintain the position of animals while radiographic images are taken.

Animal research based on the removal of the masseter muscle or surgical denervation has shown an overall reduction in mandibular dimensions, especially in ramus height. An opening of the gonial angle and localised changes in the skeletal insertions of the masseter muscle have also been shown. Experimentally-induced masseter muscle atrophy replicates the muscular weakness often seen in neuromuscular diseases in humans, such as Duchenne and myotonic muscular dystrophy, both of which display characteristic craniofacial skeletal features associated with weaker orofacial musculature, or in patients with congenital absence of the facial nerve, such as in Moebius syndrome. Clinical observations in humans have shown that there is a relationship between weaker muscular bite force and increased underlying vertical facial dimension. In contrast with the effects of surgical denervation or muscle removal, the administration of anabolic steroids such as growth hormone and testosterone are reported to have had a positive effect on the growing craniofacial region.

β2-adrenoceptor agonists (β2-agonists) were first developed to promote bronchodilation for asthmatic patients, but they are also acknowledged to have muscle growth-promoting effects, similar to those of anabolic steroids. The administration of β2-agonists has previously been shown to retard atrophy in denervated muscles. Clinical trials have highlighted the possible administration of a systemic β2-agonist for the treatment of various neuromuscular disorders, including muscular dystrophy, to improve muscle strength. Due to its high lipophilicity, a more recently synthesised β2-agonist, formoterol, has been shown to have an increased duration of action, as well as increased β2-adrenoceptor selectivity compared with traditional β2-agonists (such as clenbuterol).

The intramuscular administration of β2-agonists allows site-specific drug delivery and may minimise the deleterious cardiac effects that often accompany the systemic administration in the treatment of asthma. To date, however, the skeletal and muscular effects of intramuscular administration of β2-agonists on the masseter muscle have not been widely reported. Therefore, the present study was designed to assess the effects of masseter muscle denervation, with or without the administration of a β2-agonist, on the dentofacial complex of the growing rat.

**Materials and methods**

All experiments were approved by the Animal Experimentation Ethics Committee of the University of Melbourne (UM) (AECC number 0704146.1) and the Howard Florey Institute (HFI) Animal Experimentation Ethics Committee (AEC number 07-067). All procedures were performed in accordance with the guidelines for The University of Melbourne Animal Welfare Committee and the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (2004).

**Animals**

Thirty-one four-week-old (70–140 g) Sprague-Dawley rats were housed for a period of eight weeks in standard cages within a pathogen-free environment in the Biological Research Facility at the University of Melbourne. The animals were kept under a 12:12 hour light-dark cycle (light 0600–1800) with free access to food (rat chow) and water ad libitum. All rats were randomly assigned to either Surgical Sham (N = 5), Denervated only (N = 9), Denervated + β2 agonist (N = 8), or β2 agonist only (N = 9) groups.

The Sprague-Dawley rat displays a known growth pattern and acknowledged motor movements and behavioural traits, including normal eating, drinking and grooming. Male rats were used because the male muscles are generally larger and easier to dissect. Four-week-old rats were chosen, as the rat pups are usually weaned at 21 to 28 days. The subsequent period, from 4 to 12 weeks, is a period of rapid growth, during which the rat is normally expected to double or triple in weight.

**Experimental procedure**

The rats were anaesthetised with an intra-peritoneal (i.p) injection of a mixture of ketamine (225 mg/kg) and Xylazine (30 mg/kg), with supplemental doses administered, as necessary, to maintain an appropriate depth of anaesthesia, so that animals did not respond to tail or toe pinching.
The process of dissection was performed under a ×20 magnification stereomicroscope (World Precision Instruments Inc., Fl, USA). The left side of the rat was arbitrarily chosen as the experimental side in all animals. In the Surgical Sham and denervated groups, a small 3 to 10 mm incision was made over the area below and directly parallel to the zygomatic arch, between the eye and ear. The platysma and masseter muscle fibres were gently parted and the masseteric nerve was identified as it passed near the sigmoid notch of the mandibular coronoid process. A 5 mm section of the masseteric nerve and its branches were surgically removed en masse from the rats assigned to the denervated groups. The masseteric nerve was exposed, identified but not cut in animals from the Surgical Sham group. In animals from the Denervated + β2 agonist group, the denervated masseter was injected i.m with formoterol (100 μm in saline; AstraZeneca, Molndal, Sweden). The incision was closed with a black silk suture and a 4-0 needle. Surgery was not performed on the β2-agonist only group. All rats recovered from the anaesthesia and rehydrated with subcutaneous isotonic saline, while their temperatures were monitored and maintained with a warming pad. Following recovery, the animals were returned to their cages and observed closely. Only wet mash and water was provided to the animals on the first day, after which, regular food was supplied. Every three days, the β2-agonist only and Denervated + β2-agonist groups were given a subcutaneous local injection of formoterol (100 μg in saline) into the left masseter muscle, for a total period of eight weeks.

To keep the animals still during the intramuscular injection, each was lightly anaesthetised. Initially, animals were placed in a clear plastic drop box, ventilated with 5% Isoflurane ((1 ml/ml) distributed by CENVET Australia) in a 1:1 mix of medical grade air and oxygen. Once anaesthetised, the rat was removed from the jar and a modified nose cone was placed over its snout to maintain anaesthesia via an inhaled-gas machine that supplied the animal with 2.5% Isoflurane (0.5 L/min). The animals were anaesthetised for approximately two to three minutes, which was long enough for the intramuscular injection of formoterol to be given. The animals were monitored until full recovery had been achieved.

At sacrifice, the rat was decapitated, skinned and carefully defleshed as much as possible until the skull remained. The skull was digitally radiographed with size #4 AT/K Scan X phosphor storage plates and scanner (Air Techniques Inc., NY, USA) at a standard anode-film distance of 25 cm in a custom-made polystyrene head and film holder. A Planmeca ‘intra’ x-ray machine (Planmeca Inc., IL, USA) was used to expose the phosphor storage plates, with the following settings used for all rats: 63 kV, 8 mA and exposure time of 0.2 seconds. All phosphor storage plates were scanned at high resolution (2872 × 3816 pixels). A standardised aluminium measurement gauge was placed on each radiographic film with holes cut at 1 cm intervals along the entire film length. Three views were taken for each rat: a lateral view, with ear rod placed to transect both external auditory meati of the rat at 90° to the film and x-ray source; a submento-vertex view with the skull placed flat in the supine position; and right and left disarticulated hemi-mandibles placed flat on each radiograph (Figure 1).

Digitised radiographs were stored in DICOM format

![Figure 1. Examples of radiographs of rat skulls: (A) Submento-vertex view. (B) Lateral cephalometric view. (C) Hemi-mandibular view. Note on the hemi-mandibular view: the difference between the experimental side hemi-mandibles from a denervated animal (upper) and a β2-agonist only animal (lower).](image-url)
and examined using the Adobe Photoshop CS3 v 10.0.1 software (Adobe Systems Inc., CA, USA) at 33.3% to 50% magnification. Brightness and contrast of the digital images were altered to provide the clearest possible picture of the radiographs. Sixteen measurements (3 angular and 13 linear), previously described in studies of craniofacial growth, were taken (Table I, Figure 2). All measurements were performed in a single-blinded manner.

### Error of the Method

To determine error of the cephalometric measurement, duplicates of the 16 measurements from a random sample of radiographs were performed twice, 2 weeks apart. A paired sample t-test was used to determine if there was a significant difference between the first and second measurement sets. A Pearson correlation was calculated to determine if a relationship existed between the error of measurement and the magnitude of the measurement.

### Table II. Facial landmark definitions.

| Cephalometric measurement (cm) | Description                                                                 |
|--------------------------------|------------------------------------------------------------------------------|
| 1                              | Total skull length, Po – A                                                  |
| 2                              | Total face height, N – Pog                                                  |
| 3                              | Sagittal diastema, premaxilla – incisor, Bu – U1                            |
| 4                              | Upper face height, posterior height of snout, viscerocranium, N – U1         |
| 5                              | Anterior height of snout, viscerocranium, A – Pr                            |
| 6                              | Lower face height, U1 – Pog                                                 |
| 7                              | Mandibular angle, N – Po – Pog                                              |
| 8                              | Mandibular plane angle, N – Po/Gn – Pog                                     |
| 9                              | Total interzygomatic width (greatest posterior curvature)                    |
| 10                             | Zygomatic arch, outside greatest curvature, to the midline, (a) LHS (b) RHS |
| 11                             | Intercondylar width, condyle – condyle                                      |
| 12                             | Total length of bony mandible Go – b1                                       |
| 13                             | Height of ramus, S – Go                                                     |
| 14                             | Mandibular height, inferior border of mandible – superior condyle           |
| 15                             | Lower ramus height, L (mandibular foramen) – Go                             |
| 16                             | Hemi-mandibular plane, C – Go – Pog                                         |

Figure 2. Cephalometric measurements taken from three radiographic views from each rat: (A) Submento-vertex view. (B) Lateral cephalometric view. (C) Hemimandibular view. Measurements adapted from previous work.
sample of five rats were taken at two different time points, one week apart. Dahlberg error\textsuperscript{64} was calculated for all measurements and paired t-tests used to compare measurements at the two time points. When compared, the Dahlberg range of error was 0.02 to 0.10 mm for linear measurements, and 0.34 to 0.63° for angular measurements, which was considered small. No statistically significant differences were found between the two sets of measurements following the use of the paired t-test.

**Statistical analysis**

All values are expressed as mean ± standard error of the mean unless otherwise specified. Experimental groups were compared with each other to determine significant differences using a one-way analysis of variance for the effects of sham surgery, formoterol administration and surgical denervation, or paired t-tests to compare left and right sides with each other (SPSS v 16 for Windows, SPSS Inc. Chicago, IL, USA).

**Results**

**General visual observations**

Denervation resulted in significant atrophy of the experimental side muscle, and the animals had a characteristic longer and thinner appearance to their faces. β2-agonist administration caused significant hypertrophy of the masseter muscle and obviously shorter and broader heads in width and height. This was observed in the live and posthumously skinned animal heads. The overall body size of the animals was not noticeably different in any of the groups. The skulls of the Denervated only group also showed a mild skeletal asymmetry from the sagittal plane and skewed towards the denervated side (Figure 3).

**Differences between the surgical sham and experimental groups**

The mean final experimental-side cephalometric measurements for the surgical sham group and the three experimental groups are presented in Table II. The table indicates that, in relation to the final mean measurements for the surgical sham group, there were the following significant differences ($p < 0.05$):

- mean decreases in total skull length (measurement 1) of 2.3% and 2.8%, respectively, for the Denervation + β2-agonist and β2-agonist only groups;
- mean decreases in the sagittal diastema (measurement 3) of 5.9% and 4.8%, respectively, for the Denervation + β2-agonist and β2-agonist only groups;
- a mean decrease in total inter-zygomatic width (measurement 9) of 3.8% for the Denervation only group (Figure 4);
- a mean decrease in the left zygomatic arch width to the midline (measurement 10a) of 4.5% for the Denervation only group;

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Figure3.png}
\caption{View of defleshed skulls: (A) View from above, showing a β2-agonist only specimen on the left and Denervated only specimen on the right. Note the difference in inter-zygomatic width transverse dimensions. In the Denervated specimen, there is also a slight skeletal asymmetry towards the experimental (left) side. (B) Left hemi-mandibles from a Denervated specimen (above) and a β2-agonist only specimen (below). Note the difference in size of the angular process and the condyle, the total height of the ramus, and the lower ramus height between the two specimens.}
\end{figure}
• a mean decrease in total bony length of the hemi-mandible (measurement 12) of 4.8% for the Denervation only group;

• a mean decrease in the hemi-mandibular height of the ramus (measurement 13) of 10.3% for the Denervation only group;

• mean decreases in total hemi-mandibular height (measurement 14) of 11.3% and 7.5%, respectively, for the Denervation only and Denervation + β2-agonist groups;

• a mean decrease in lower hemi-mandibular ramus height (measurement 15) of 11.8% for the Denervation only group;

• mean increases in the angle of the hemi-mandible (measurement 16) of 7.2% and 3.7%, respectively, for the Denervation only and Denervation + β2-agonist groups (Figure 5).

**Differences between control and experimental sides in all groups**

The mean final skeletal cephalometric measurements for experimental (left) and control (right) sides in the four groups are presented in Table III. The table reveals that, in comparison with the mean control side measurements, there were the following significant differences ($p < 0.05$) on the experimental sides:

• a mean decrease in total mandibular bony length (measurement 12) of 3.5% in the Denervation only group;

• mean decreases in the height of the mandibular ramus (measurement 13) of 2.7%, 9.0% and 5.2%, respectively, in the Surgical sham, Denervated only and Denervated + β2-agonist groups;

• mean decreases in total height of the mandible (measurement 14) of 7.5% and 5.6%, respectively,
in the Denervated only and Denervated + β2-agonist groups;

- mean decreases in lower ramus height (measurement 15) of 8.3%, 12.4% and 5.9%, respectively, in the Surgical sham, Denervated only and Denervated + β2-agonist groups;

- mean increases in the angle of the mandible (measurement 16) of 4.5% and 3.5%, respectively, in the Denervated only and Denervated + β2-agonist groups (Figure 5).

Discussion

Mandibular plane angle increase following denervation of the masseter muscle

The results of previous studies have shown that significant changes can occur in the underlying facial skeleton following the removal of the masseter muscle or denervation of the masseteric nerve in the rat. Moore believed that these growth differences in the mandible may arise from the post-surgical reduction in mechanical stresses generated by the muscles. An average increase of 6° in the mandibular plane angle in experimental animals compared with controls has been reported to follow masseter removal. A similar result was found in the present study, in which an average 7° increase was observed in the mandibular angle following denervation of the masseter in the growing animals. This would support the results of earlier studies of denervation in rats and primates, in which weakened muscles resulted in more vertical growth patterns and an increase in the mandibular plane angle.
ALTERATION OF MASSETER FUNCTION

Ramal height decrease following denervation of the masseter muscle

The results of the present study are consistent with a previously-reported decrease in ramal height of 10% following the removal of the masseter muscle in rats,\textsuperscript{37} as well as those reported by Carter and Harkness,\textsuperscript{18} who also found significant changes in ramal height. The features of increased gonial angles and decreased ramal height correspond with the facial skeletal features of human subjects presenting with dolichocephalic patterns.

\textbf{β2-agonist administration}

It has previously been shown that, following β2-agonist administration in non-surgical subjects, muscle volume increased on average by 50%, muscle mass on average by 36%, and muscle fibre cross-sectional area on average by 29%.\textsuperscript{65} In contrast, following surgical denervation of the masseter muscle, muscle volume decreased on average by 56%, muscle mass on average by 38%, and muscle fibre cross-sectional area on average by 38%. Despite these likely significant increases or decreases in muscle mass, no equivalent positive or negative skeletal effects were found in the present study.

Overall facial skeletal changes following denervation of the masseter muscle

The skeletal effects of denervation of the masseter muscle in the present study were limited to the mandible and the transverse dimension of the skull. Significant decreases in the dimensions of the mandible were observed as the experimental hemimandible had, on average, a shorter overall length and a decreased ramal height, especially in the area below the inferior alveolar canal. This is a significant area of insertion of the masseter muscle and also where the largest amount of height reduction was observed. Surgical denervation also resulted in statistically significant skeletal effects seen from the submentovertex view of the skull, with an average reduction in total inter-zygomatic width of 3.8%, especially on the experimental side. This is consistent with the general visual observation of a decrease in skull width and the results of previous studies which related muscle function to transverse skull dimensions in growing rats.\textsuperscript{69} It was noteworthy that the administration of the β2-agonist into the denervated masseter muscle resulted in only a small average decrease in total skull length and a decrease in the sagittal diastema, which was consistent with the general observation that the final rat heads were shorter in the antero-posterior dimension and wider in the transverse dimension than those of the controls. The results of previous studies suggested that, despite formoterol being administered \textit{i.m}, it also seemed to have a local effect on other muscles in the craniofacial region, with significant increases seen especially in the mass and volume of the contralateral masseter muscle.\textsuperscript{66,67}

\textbf{Myostatin gene control of muscular development}

It is generally accepted that the functioning muscles have a significant morphogenetic effect on the skeletal tissues to which they are attached. The myostatin gene is a potent negative regulator of muscle development, and a deficiency in the gene induces a dramatic increase in skeletal muscle mass resulting mainly from muscle hyperplasia and partly from hypertrophy. Myostatin-deficient mice have been shown to produce relatively greater bite forces and to have, on average, 56% larger masseter muscles than controls.\textsuperscript{22,70}

Other possible effects on bone of β2-agonist administration

The fact that there were only minor skeletal changes following only β2-agonist administration may be partly explained by a parallel alteration in the quality of the bone, such as increased density or cortication,\textsuperscript{65} which has previously been demonstrated in mice.\textsuperscript{71} There have been reports of the possible effects of β2-agonists as β-receptors exist in bone,\textsuperscript{72} and may influence bone growth by decreasing bone mineral content in growing rats.\textsuperscript{73} β2-agonists may induce alterations in the bony architecture and mechanical properties, opposing their own anabolic action and, in effect, negating the musculoskeletal interaction.

\textbf{Surgical Sham group}

An interesting finding of the present study was that, in the Surgical Sham group, significant differences were found between the sham experimental and control sides for ramus measurements (CM 13 and CM 16). It is possible that damage to the muscle or early post-operative inflammation and pain may have
limited muscular function during the experimental period and influenced underlying skeletal growth. This result was interesting because the Surgical Sham animals underwent massteric nerve isolation, but not denervation. Surgical denervation is reported to remove approximately 70% of the muscle spindle afferent fibres from all masticatory muscles on the experimental side, and yet proprioceptive information from the skin, joints, teeth and remaining muscle spindle fibres is still acknowledged to provide feedback to support normal patterns of muscle activity. The masseter muscles contribute about 54% of the total masticatory muscle mass in the rat, and, following denervation, the remaining masticatory muscles may compensate for any loss of function. These observations may, therefore, imply that a possible neurological mechanism is involved in bone remodelling and adaptation of the bone to the muscle.15 Such ‘neurotropic’ regulation would also integrate with Moss’s Functional Matrix Theory, in which nerves supply almost all so-called ‘capsular’ and ‘periosteal’ functional matrices.

Use of formoterol in treatment of muscle-wasting diseases

That local administration of the β2-agonist had a negligible effect on the underlying skeleton may have implications for the treatment of sarcopenia or muscular dystrophy in growing patients, because iatrogenic craniofacial side-effects may be avoided.76,77 Previous treatments for these disorders have included the administration of growth hormone, anabolic steroids and testosterone, all of which have been associated with unwanted effects on the growing craniofacial skeleton.50,78 If the administration of formoterol has little effect on skeletal growth in the human craniofacial region, it may be useful in the treatment of muscle wasting conditions such as Myotonic Dystrophy.

Many factors may contribute to or influence craniofacial skeletal growth. The level to which each factor might contribute, however, is not well understood and has so far been difficult to measure and may never be fully understood. Other factors, such as ‘neurotropism’ or gene expression, may also contribute to overall craniofacial size and shape. Therefore, clinicians are advised to consider the mandibular muscles and their possible effects79,80 when planning and carrying out routine orthodontic treatment in growing patients.

Conclusions

Taking into account the limitations of any animal laboratory study, the following conclusions may be drawn:

1. An increase in muscle size and mass following the administration of the β2-agonist, formoterol, had only limited effects on changes occurring in the underlying skeletal structures during growth.

2. Denervation of the masster muscle was associated with atrophy of the muscle itself, and significant skeletal changes in the craniofacial area during growth.

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ALTERATION OF MASSETER FUNCTION

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