Masticatory retraining effect on masseter muscle, facial morphology and alveolar bone structure in the adult rat

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To my son Martin

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ABSTRACT

The aim of this series of investigations was to study the effect of masticatory muscle retraining in adult rats with an earlier reduced masticatory muscle function on the craniofacial morphology, on the internal alveolar bone structure and on the deep masseter muscle.

Material and Methods: Sixty young male rats received soft diet for a prolonged period, so that the animals developed weak masticatory muscles. After 21 weeks when the animals had nearly ceased their body growth the rats in the experimental group were divided into two groups. One group continued with soft diet until the end of the experiment (hypofunctional group). The other group received ordinary hard food to get the possibility to retrain their masticatory muscles (rehabilitation group). A third group (control) received ordinary hard food during the whole experimental period (27 weeks). Morphometric analysis of the mandible, cephalometric analysis of the skull, microtomographic histomorphometry (μCT) of the alveolar process of the mandible and quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) on "muscle biopsies" were performed.

Results: The rehabilitation group was only marginally different compared to the hypofunctional group concerning the lateral view morphology of the mandible, although a general tendency to approach (catch-up) the normal group was observed. The variables under study concerning the trabecular bone in the rehabilitation group also showed a catch-up towards the control group. The increase of the anterior zygomatic arch width and interzygomatic width were slightly larger in the rehabilitation group compared to the other groups. The gene protein expression of MYH 3 and MYH 7 were significantly higher in the rehabilitation group compared with the other groups.

Conclusions: The increased functional demands seem to influence the craniofacial morphology in adult rats at areas under direct influence of the masticatory muscles. Alveolar trabecular bone architecture did improve after functional rehabilitation although the negative effects of hypofunction were not completely reversed. Muscular retraining induced genetic expression of the slow contracting (MYH 7) isoform levels and embryonic (MYH 3) isoform to withstand increased masticatory mechanical load.

Keywords: Adult, rat, craniofacial morphology, alveolar trabecular bone, MYH, mandible, masticatory function

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SAMMANFATTNING PÅ SVENSKA

Man vet sedan tidigare att en muskelsvaghet kan påverka bettutvecklingen och ansiktsskelettet under tillväxten. De finns kunskapsluckor i vad som händer med bettet och ansiktsskelettet om man har en försämrad tuggfunktion och svag tuggmuskulatur i vuxen ålder, men får en möjlighet att förbättra tuggfunktionen, till exempel efter ortognat kirurgi eller behandling med implantat retinerade protetiska konstruktioner.

Syftet med denna avhandling är att studera effekten av träning på en försvagad tuggmuskultur hos vuxna råttor. Dels effekten på tuggmuskeln i sig, men även påverkan på skallen i transversell led, formen av mandibeln samt det spongiösa benet i mandibelns alveolar utskott.
Material och Metod: En experimentell grupp bestående av unga råttor fick mjuk kost för att framkalla en svag tuggmuskel. När råttorna nått vuxen ålder delades gruppen i två delar. Den ena gruppen fortsatte med mjuk kost, medan den andra gruppen övergick till hård kost (pellets) för att träna sina tuggmuskler under sex veckor. Parallellt under studien fanns en kontroll-grupp som åt hård kost. Råttorna röntgades och mätningar utfördes på röntgenbilderna. Mandibeln fotograferads med standardiserad metod och på dessa utfördes morfologiska mätningar. Mandibelns alveolar utskott skikt-röntgades och analyserades. Från masseter muskeln togs vävnadsprover vilka analyserades med hjälp av qPCR (Quantitative polymerase chain reaction).

Resultat: Träning av försvagade tuggmuskler gav en ökning av alveolarutskottets och okbenets bredd, men gav endast en mindre förändring av mandibelns form i förhållande till de djur som fortsatte med mjuk kost (svag tuggmuskel). Det spongiösa benet i mandibeln påverkades av träningen, men nådde inte fullt ut till samma mätvärden som kontrollgruppen. I masseter muskeln blev det en förändring av gen sammansättningen. Transkriptionen av generna MYH 3 och MYH 7 ökade i träningsgruppen i förhållande till de andra två grupperna.

Slutsats: Träning av försvagad tuggmuskulatur hos vuxna råttor ger en påverkan på ansiktsskelettet framförallt där tuggmusklerna fäster. Förändringen i sammansättningen av gener i tuggmuskeln är med största sannolikhet för att klara av den ökade belastningen som muskeln blir utsatt för.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Ödman A, Mavropoulos A, Kiliaridis S. Do masticatory functional changes influence the mandibular morphology in adult rats. *Arch Oral Biol. 2008 Dec;53 (12):1149-54.*

II. Ödman A, Bresin A, Kiliaridis S. The effect of retraining hypofunctional jaw muscles on the transverse skull dimensions of adult rats. *Accepted for publication in Acta Odontol Scand.*

III. Mavropoulos A, Ödman A, Ammann P, Kiliaridis S. Rehabilitation of masticatory function improves the alveolar bone architecture of the mandible in adult rats. *Bone 2010 Sep;47(3):687-92.*

IV. Ödman AM, Hunt NP, Matloub Moawad HA, Sinanan AC, Kiliaridis S, Lewis MP. Molecular changes in detrained & retrained adult jaw muscle. *Eur J Orthod. 2013 Oct;35(5):659-63.*

CONTENT

ABBREVIATIONS

Tb.N.	Trabecular number
Tb.Th.	Mean trabecular thickness
BV	Bone volume
CBCT	Cone beam computed tomography
cDNA	Complementary deoxyribonucleic acid
EMG	Electromyographic
mRNA	Messenger ribonucleic acid
MyHC	Myosin heavy chain
sEMG	Surface electromyographic
SN-GoGn	SellaNasion-GonionGnation
TV	Total volume
VOI	Volume of interest
qPCR	Quantitative polymerase chain reaction

DEFINITIONS IN SHORT

Bone volume fraction	Bone volume/total volume ratio
Bone surface ratio	Bone surface/total volume ratio
Trabecular thickness	Mean thickness of trabeculae
Trabecular number	Number of trabeculae per unit length
Connectivity density	Number of trabeculae per unit volume
Trabecular separation	Mean distance between trabeculae

INTRODUCTION

Edentulism in adulthood is associated with a reduced functional capacity of the masticatory system. Similarly, adults with severe maxillofacial discrepancies can have a reduced masticatory performance due to few occlusal contacts and low bite force. However, an improvement of the functional capacity of the masticatory system may occur in edentulous adults who receive implant-retained prostheses or in adults who undergo orthognathic surgery to treat their maxillofacial discrepancies. It is though unknown if the improvement of the functional conditions of the masticatory system in those subjects may affect their alveolar bone structure and the craniofacial morphology and to which extent.

A better insight of the effects of increased masticatory muscle function in adulthood may help us to understand the possibility and extent of a potential skeletal adaptation in the craniofacial region in adulthood. These effects may influence the stability of the treatment outcome of combined orthodontic and orthognatic surgery or have an effect on the overall morphology of the alveolar process after insertion of implants.

BACKGROUND

Edentulism and masticatory muscle functional capacity

Edentulism in adulthood is associated with a reduced functional capacity of the masticatory system (Ingervall and Hedegård 1980, Raustia et al. 1996, Fontijn-Tekamp et al. 2000). Tallgren et al. (1980) showed that the EMG activity of the jaw closing muscles (masseter muscle, temporalis muscle and digastric muscle) at hard bite in partially edentulous patients is lower than in patients with a full dentition. A long period of edentulism not only gives a reduced functional capacity of the masticatory system i.e. reduced EMG activity could also result in a change in the density of the masseter muscle in computed tomography implying muscle atrophy (Raustia et al. 1996). This atrophy may be the cause of a low bite force in edentulous individuals when compared to dentate subjects. Furthermore, Newton et al. (2004) found that the cross-sectional area of the masseter was smaller in edentulism patients compared with dentated.

Edentulism and its effects on facial skeleton

Edentulism in adult age gives a decrease in facial height that is due to a forward-upward rotation of the mandible (anterior autorotation), because of the loss of teeth and a reduction of the height of the alveolar process (Tallgren 1967). The level of resorption of the alveolar bone is highest in the lower arch. The resorption of the lower anterior ridge is four times more than

for the upper anterior ridge (Tallgren et al. 2003). Edentulism not only leads to a change in the vertical inter-maxillary relation due to the autorotation of the mandible, but also affects the form of the mandible. The change of the mandibular morphology in edentulous individuals occurs by a decrease in height of the condyle (Huumonen et al. 2010) and ramus (Huumonen et al. 2010, Oksayan et al. 2014). Furthermore, the gonial angle increases (Engström et al. 1985, Huumonen et al. 2010) due to decrease activity of the pterygomasseteric sling causing bone resorption of the gonial process (Raustia et al.1997).

Severe craniofacial discrepancies / malocclusions and muscle functional capacity

Severe maxillofacial discrepancies leading to malocclusions as open bite and severe class II or III relation may lead to a reduced number of occlusal contacts. It is worth to remember that the number and size of occlusal contacts influence the masticatory performance, as they determine the capacity of processing the bolus (Owen et al. 2002). Abrahamsson et al. (2015) concluded that severe open bite had a negative effect on the masticatory performance. English et al. (2002) found that both class II and III malocclusion had a negative effect on their chewing capacity, like for example chewing a raw carrot. Individuals with class III malocclusion had the greatest difficulties. This can be due to few occlusal contacts and a lower masticatory capacity. Individuals with an open bite or a class III malocclusion have less maximum isometric bite force compared with controls (Proffit et al. 1983, Ellis et al. 1996).

There is a correlation between the sagittal skeletal base relationship and the

sEMG (surface electromyographic) activity of the masseter and temporalis muscle. The activity was higher for individuals with a neutral or low angle (SN-GoGn) compared to a high angle relationship (Tecco et al. 2007, Ueda et al. 2000).

Individuals with a deep bite and full occlusion have a different composition of their fibre types in their masticatory muscles compared with individuals that have an open bite and a poor occlusion. Patients with short lower face, often seen at deep bite, have an increase of type II fibres and long lower face cases, often seen at open bite, on the other hand have a decrease of type II fibres and also a smaller fibre size. Type II fibres are fast acting fibres. These fibres produce maximum force during a short period of time as for example when clenching of teeth. Open bite cases have a greater number of type I fibres (Rowlerson et al. 2005).

Gedrange et al. (2005) found a difference in the mRNA MyHC composition between class II and class III cases before surgery. In the anterior part of the masseter muscle of patients with class II contained more MyHC type I and IIx compared with class III patients. Concerning the myosin heavy chains in patients with long face the fast isoforms are reduced compared to individuals with normal vertical relations. (Hunt et al. 2006). The MyHC (myosin heavy chain) is the important contractile protein in skeletal muscle fibres. The interaction between the rod-like myosin molecules (myosin heavy chain) and the globular-like actin molecules is one of the major mechanisms responsible for muscular contraction (Pitman and Peterson 1989).

Rehabilitation of edentulism

Edentulous patients who receive implant-retained prostheses get higher muscle activity and bite force, and by this an improved chewing efficiency compared to non-treated patients. (van der Bilt et al. 2006, Jemt et al. 1996, Müller et al. 2012). Enkling et al. (2016) measured the maximum voluntary bite force with an occlusal force meter in their study on edentulous patients that who got implant retained overdentures. The bite force increased immediately and continued to increase during the follow-up period that lasted for a year.

Apart the effect of the rehabilitation of masticatory muscles an influence on the bone re-modeling seems to occur since masticatory loads on implants during a period of ten months resulted in more extended bone-implant contact compared to unloaded implants in an experimental study in dogs (Berglund et al. 2005).

Rehabilitation of severe craniofacial discrepancies and malocclusions

After orthognatic surgery there is an improvement of the quality and efficiency of the occlusion i.e. increased number of occlusal contacts (Harzer et al. 2007, Di Palma et al. 2009). This in turn can lead to greater muscle forces (Breuel et al. 2013). The masticatory muscles adapt to the new conditions that occur after orthognatic surgery. The individuals often reach the same strength in their masticatory muscles as individuals without severe malocclusions (Ellis et al. 1996, Throckmorton et al. 1996, Proffit et al. 1989). When a skeletal jaw muscle adapts to new demands the fibre-type

composition changes (Adams et al. 1993, Oishi et al. 1998). The change of fibre-type composition is made possible by switching on one subset and repressing another subset of genes (Goldspink et al. 1992). Fibre-type changes normally follow a strict order: from slow fibre-type towards fast and then towards faster (I (slow) →IIA → IIX → IIB (fastest)) and vice versa (Schiaffino and Reggiani 1994).

Harzer et al. (2007) collected biopsies before and six months after orthognatic surgery from patients that were undergoing surgery to correct mandibular prognathism or retrognathia (class II and class III). The mRNA MyHC analysis that they performed showed a significant shift in the relative content from type I (46% before, 37% after) to type IIa (29% before, 42% after) but without significant differences between individuals treated for retrognathia or prognathism. They also found a correlation between the shift of MyHC and the increased number of occlusal contacts. This can be due to a better stability and the increased pair of occluding teeth that can withstand the higher forces.

Studies have been performed with the use of CBCT (cone beam computed tomography) to study skeletal changes after orthognatic surgery (surgery due to class II or class III malocclusion). The follow up periods were between one and three years. (Carvalho et al. 2010, De Paula et al. 2013, Franco et al. 2013). One year after mandibular advancement a change in the posterior border of the ramus was significantly affected (Carvalho et al. 2010). De Paula et al. (2013) studied the effect one year after orthognathic surgery for class III malocclusion and found statistically significant changes in the ramus, chin and on the condylar surface. Franco et al. (2013) found that three years after mandibular advancement 20% of the patients had an adaptive bone remodeling in the chin, ramus and condylar region. In all studies there

was a large individual variability. It is however unclear whether those changes represent relapse after surgery/orthodontics or signs of bone adaptation due to improved muscular function or effect of the stretched tissues.

Those observations on muscles and facial skeleton adaptation were made in adult subjects in non-experimental settings where the degree of change in masticatory functional demands may vary due to individual variations.

In experimental animal studies on the other hand genetic variables and masticatory muscle demands can be controlled.

Animal models and methods to study rehabilitation (retraining) of hypofunctional masticatory muscles

To be able to answer some of those questions we needed an experimental model that allowed us to re-train the masticatory muscles in adult animals with hypofunctional muscles.

Various models have been applied to produce changes in the masticatory muscle function. Surgery to denervate or resect masticatory muscles, Botulinum injection or changing the consistency of the diet (soft diet instead of normal hard pellet diet) are some examples of different models that have previously been used to study the effect of masticatory function on the skull during growth (Horovitz and Shapiro 1995, Kiliaridis and Shyu 1988).

To denervate or resect masticatory muscles are non-reversible methods. The effect of Botox (Botulinum) injections are shown by Fortuna et al. (2013) not to disappear for at least 6 months in skeletal muscles and thus not an optimal experimental method due to the time span and ageing of the animal.

Changing the consistency of the diet is however a non-invasive method of affecting the muscular function and a reversible method. Another advantage of the model is that it makes it possible to alter muscular function in an anatomically and functionally intact masticatory system. Therefore, it allows to induce hypofunction in masticatory muscles that can be followed by muscular retraining, which is why it is the method of choice for this study.

Our hypothesis is that retraining of masticatory muscles in adulthood may have effect on the masseter muscle, the alveolar internal bone structure and on the outer bone facial morphology at sites that are under direct muscle loading.

An experimental set-up was chosen to study if retraining of the masticatory muscles at adult age may affect the craniofacial morphology of rats, which had reduced masticatory functional demands during growth.

AIM

The general aim of the present thesis is to investigate the effect of masticatory functional changes on the craniofacial morphology of adult rats, on the internal alveolar bone structure and on the masticatory muscles (will be represented here by the masseter muscle).

The specific aims were to investigate the effect of retraining of hypofunctional jaw muscles in adult rats on:

- the morphology of the mandible on the sagittal plane, especially in areas of masticatory muscle insertions;
- the transverse cranial dimensions;
- the morphology and the trabecular architecture of the mandibular alveolar bone;
- the expression of the gene coding for the contractile protein of the deep masseter muscle.

MATERIALS AND METHODS

Animals (Paper I-IV)

The experiments were carried out on male Sprague Dawley rats. Sixty rats were used in papers I, II and III. Thirty-seven of them were used in paper IV. At the start of the experiment the rats were approximately 21 days old. The rats were fed and watered ad libitum.

Diet (Paper I-IV)

The rats received a hard diet (ordinary pellets for rats) or a soft diet. The soft diet was made of finer form of ordinary pellets (R34, Lactamin, Södertälje, Sweden) mixed with water in standardized proportions (2:5, R34: water).

Experimental design (Paper I-IV) *(Figure 1)*

The Ethics Committee of the University of Gothenburg, Sweden had approved the experimental protocol.

The rats were initially divided into two groups (paper I to IV). One group of rats was fed hard diet during the whole experimental period (16 rats in paper I to III and 13 of these in paper IV) (control/normal group). The other group composed of 44 rats in paper I-III and 24 in paper IV received the soft diet, so that the rats should develop weak masticatory muscles (Kiliaridis and Shyu 1988).

The group being fed soft diet for 21 weeks was divided into two equal groups (matched for weight). One group continued on soft diet (hypofunctional group), and the other group changed from soft to hard diet and designated the

rehabilitation group. Throughout the experimental period, the rats were weighed every second week to monitor their health and growth. The new condition lasted for a six week long period. At the end of the experimental period the animals were sacrificed in a CO_2 chamber. The total experimental period lasted for 27 weeks. At the end of the experimental period the mandible was dissected and separated at the symphysis and defleshed under water. Fixation was performed with ethanol 99%.

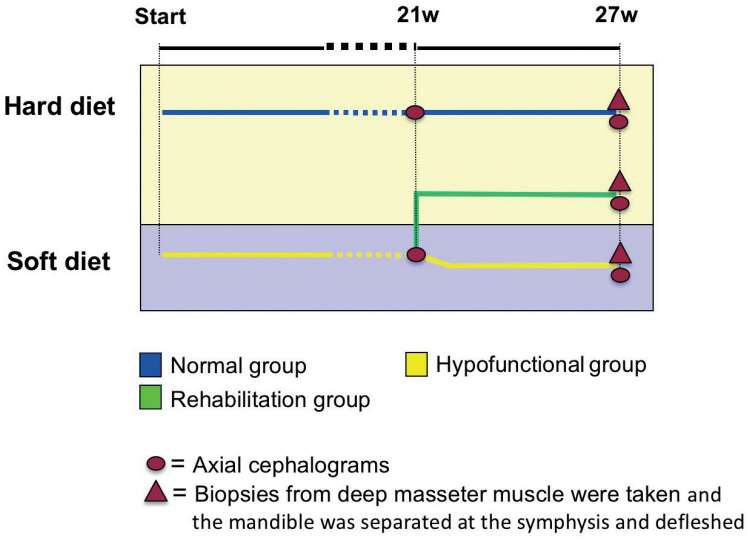

Figure 1. Experimental design

Methods

Morphometric analysis of the lateral view of the mandible (Paper I)

After fixation each left mandible was placed lying on its lingual side on top of a custom-made light source and was photographed using a digital camera fixed on a steady stand with standardized configuration settings.

The images were directly transferred to a computer for interpretation using customized cephalometric software (Viewbox 3.01; D. Halazonetis, Athens, Greece). The software permits the use of preprogrammed custom shapes (called ‘‘snakes’’ or line contours) that can be aligned with anatomical reference points and bone outlines. This procedure led to the development of a digital anatomical map where 164 anatomical and 6 derived reference points were available for measurements and calculation (Figure 2). The line defined by the mental and the mandibular foramina was used as a reference line. This method was used and calibrated in a study by Mavropoulos et al. (2004). To be able to compare their study on growing rats with this study we used the same measurements that they had chosen based on anatomical, geometrical and functional criteria.

Three mean tracings were constructed, one for each group, which were subsequently superimposed on the same reference line.

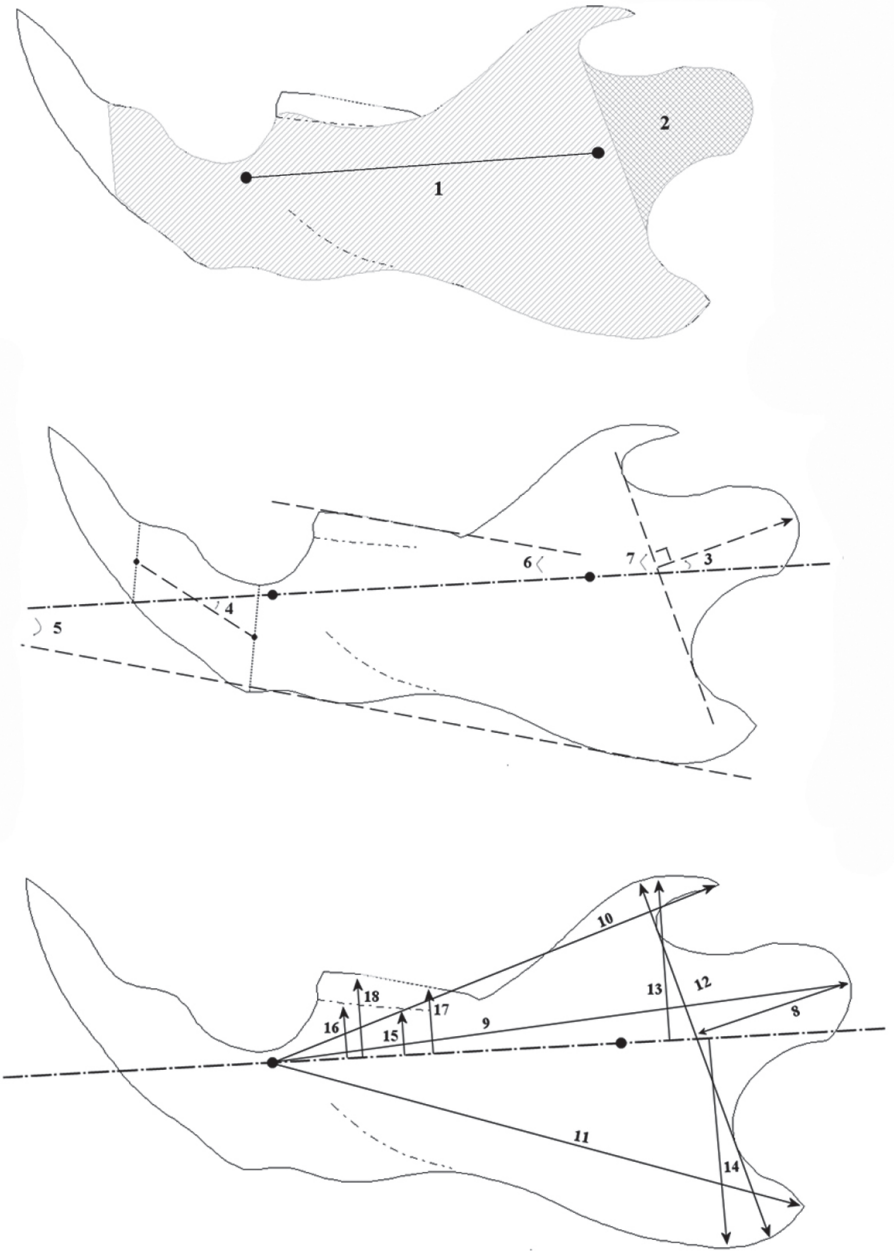
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Figure 2 The measurements under investigation: The solid black circles correspond to the mental and mandibular foramina, which define the reference line.

1. the area of the external surface of the mandible, including the condylar process, excluding the teeth
2. the area of the condylar process, delimited by the condylar tangent (tangent to both upper and lower mandibular notches)
3. the angle formed by the perpendicular from the most posterior and superior part of the condyle to the condylar tangent and the reference line
4. the angle formed by the axis of the incisor alveolar process and the reference line.(incisor alveolar axis: a line from the midpoint between the upper and lower aspect of the incisor alveolar crest to the midpoint between the deepest part of the incisor alveolar curvature and the edge of the digastric fossa)
5. the angle formed by the lower border of the mandible and the reference line
6. the angle formed by the occlusal plane and the reference line
7. the angle formed by the condylar base and the reference line
8. the maximal distance of the condyle from the condylar tangent
9. the maximal distance between the mental foramen and the condyle
10. the maximal distance between the mental foramen and the coronoid process
11. the maximal distance between the mental foramen and the angular process
12. mandibular posterior width (tangent to the upper and lower mandibular notches)
13. the maximal vertical distance of the coronoid process from the reference line
14. the maximal vertical distance of the angular process from the reference plane
15. the distance between the reference line and the deepest point of the alveolar crest of the third molar
16. the distance between the reference line and the deepest point of the alveolar

(Modified from Ödman et al. 2008)

Cephalometric analysis of the transverse dimensions of the skull (Paper II)

Axial cephalograms were taken of all the animals 21 weeks into the experiment and at the end of the experimental period (week 27). The cephalograms were performed under anesthesia. At week 27 the cephalograms were taken after the rats were sacrificed. A specially designed cephalostat in combination with a standard dental x-ray machine was used. The axial cephalograms were digitised with a flatbed scanner.

In order to measure the transverse dimensions of the skull 14 points were identified to assess anatomic regions with different functional demands (Figure 3). The points under study were partly chosen from Katsaros et al. (2002) to be able to compare with growing animals.

The cephalograms were blinded i.e. it was not known to which group the cephalogram belonged to. The registrations were performed with the image analysis program NIH-Image version 1.61 PPC. (The updated version of NIH image is Image J, at https://imagej.nih.gov/ij/)

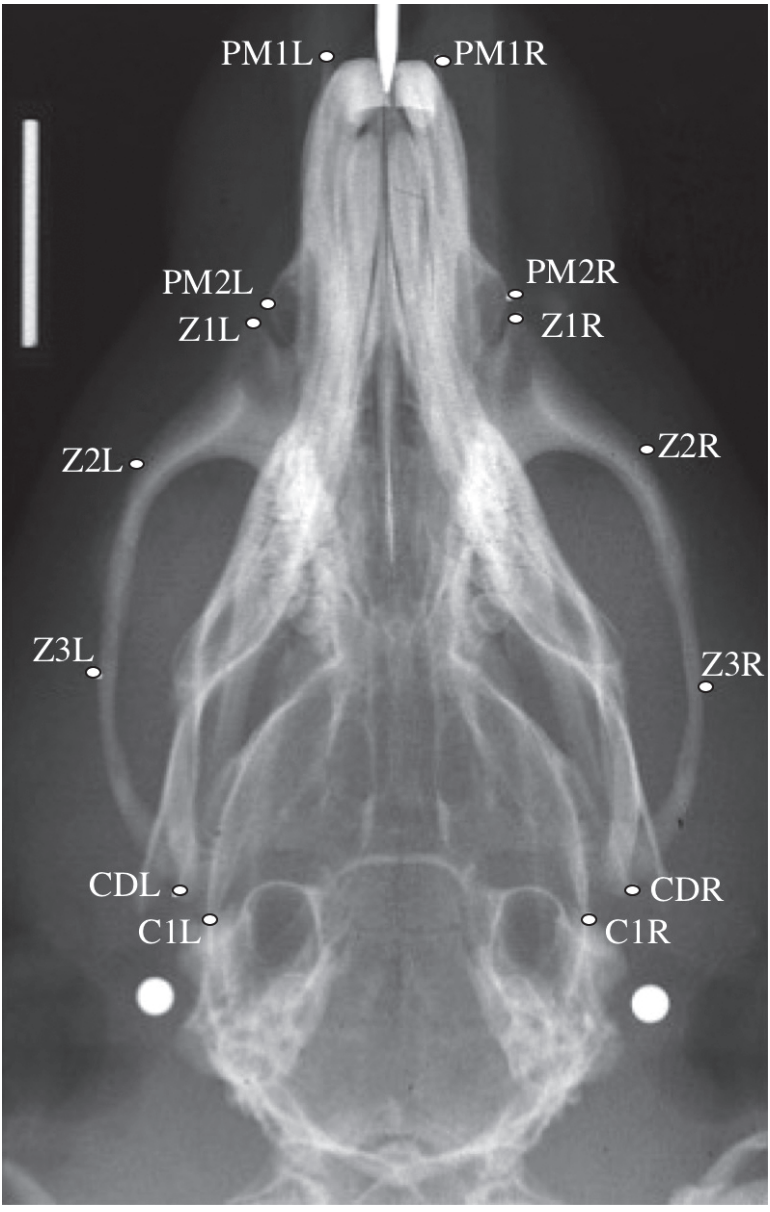


Figure 3. Measurements under study: PM1L-PM1R: the anterior premaxillary width, PM2L-PM2R: the posterior premaxillary width, Z1L-Z1R: the anterior zygomatic width, Z2L-Z2R: the anterior zygomatic arch width, Z3L-Z3R: the interzygomatic width, CDL-CDR: the intercondylar width and C1L-C1R: the posterior skull width.

Microtomographic histomorphometry by microcomputed tomography (μCT) of the mandibular alveolar process (Paper III)

To study the morphology and the trabecular architecture of the alveolar bone, the fixated mandibular halves were sawed into three pieces, to allow insertion in the machine. The middle part including the alveolar process was investigated by using microtomographic histomorphometry with a high-resolution μCT system. On the gray-scale images the volume of interest (VOI) was drawn starting from the first slice containing the crown of the first molar and moving dorsally 100 slices in the area of the alveolar process between the roots of the molars and the root of the incisor. The trabecular bone was then marked on the first and on the last slice. Each slice was inspected and the contour of the trabecular bone was modified where it was necessary. Microcomputed histomorphometric indices were calculated directly from the binarized VOI. Total volume (TV) was the volume of the whole sample examined. Bone volume (BV) and bone surface (BS) were calculated using tetrahedrons corresponding to the enclosed volume of the triangulated surface. Mean trabecular thickness (Tb.Th.) were determined from the local thickness at each voxel representing bone. Trabecular number (Tb.N.) and Connectivity (A measure of the degree of the connectivity of trabeculae normalized by TV (Volume of the entire region of interest)) density was calculated.

This method has earlier been described and calibrated by Mavropoulos et al 2004. We chose to use the same area of the mandible to be able to compare with a group of growing animals.

Morphometry of the mandibular alveolar process (Paper III)

The height and the width of the alveolar process were measured by using a caliper on the section of the mandible corresponding to the middle of the 1st molar. The width of the alveolar process was measured perpendicularly to the height at the midlevel of the 1st molar apices. The height was measured between the mandibular canal and the bifurcation of the molar.

Evaluation of the contractile protein gene expression in the deep masseter muscle (Paper IV)

At the end of the experimental period the deep masseter muscle was directly frozen in thawing isopentane (−150°C) and stored at −80°C. Ribonucleic acid (RNA) was extracted from the masseter muscle by using a FastPrep FP120A bench-top reciprocating device and RNeasy Mini Kit (Qiagen). The RNA's quality and concentration was measured and controlled with a Spectrophotometer. By using a high capacity cDNA archive kit the RNA was converted into complementary deoxyribonucleic acid (cDNA). Quantitative polymerase chain reaction (qPCR) was performed and the masseter muscle derived cDNA was targeted for sequences of MyHC genes. (*Table 1*)

Table 1: Myosin heavy chain isoforms investigated in this study.

Gene of interest	**Myosin heavy chain protein**	**Muscle contraction**
MYH 1	Myosin heavy chain IIx	Fast
MYH 2	Myosin heavy chain IIa	Faster
MYH 3	Embryonic myosin heavy chain	Fairly fast
MYH 7	Beta-cardiac myosin heavy chain, Myosin heavy chain I	Slow

Statistical analysis

In all four papers all data were represented as mean and standard deviation (mean±SD). Analysis of variance (ANOVA) was used to investigate differences between the three groups under study.

In paper III the Pearson correlation test was also applied in order to detect correlations between the morphometric and the micro-tomographic variables.

All statistical analyses were performed using SPSS statistical package (13, 16 and 21). A result was considered statistically significant at $P < 0.05$.

Error of the method

Paper I:

The whole measurement procedure was repeated in 25 of the mandibles 2 weeks after the initial measurements. The error of the method was calculated according to the Dahlberg´s formula: $Se=\sqrt{\Sigma d2/2n}$ where d is the sum of the squared differences between pairs of recordings and n is the number of duplicate measurements. It varied between 0.19 and 1.49 mm2 for area, between 0.34° and 0.73° for angular, and between 0.04 and 0.18 mm for linear measurements. No systematic error was detected between the two sets of measurements tested by using the paired t-test. The coefficient of variation (Houston 1983) was found to be below 3.15% for all measurements with the exception of ‘‘condylar process inclination’’ where it was 11.55%.

Paper II:

Double measurements were performed on twelve (a fifth of the animals) radiographs repeated after 14 days to evaluate the method error.

The magnitude of the combined method error was calculated according to Dahlberg´s formula $Se=\sqrt{\Sigma d2/2n}$, where d is the difference between two registrations of a pair and n the number of the double registrations (Dahlberg, 1940). The average random error was found to be between 0.02-0.05 mm. Paired t-tests were used between the two series of measurements to test for any systematic error; no statistically significant differences were found in this test.

Paper III:

The method used in paper III was calibrated and discussed in Mavropoulos et al. 2004. “The whole acquiring procedure was repeated another four times for four mandibles chosen at random to calculate the coefficient of variation (CV). It ranged from 1.05% to 2.25% for trabecular thickness (Tb.Th) and trabecular bone volume fraction (BV/TV), respectively.”

Paper IV:

The RNA’s quality and quantity (concentration) was controlled/measured with a Spectrophotometer before it was converted into cDNA.

Comments to the material and method

The reasons for the choice of an experimental animal model with male Sprague Dawley rats were several:

- Rats are genetically homogeneous and thanks to that the individual variations, which may exist in clinical studies are avoided.
- A large number of studies on craniofacial growth and adaptation have earlier been performed in rats, allowing easier comparison of the results.
- The method of changing the masticatory muscle function by altering the consistency of the diet has previously been applied in Sprague Dawley rats (Ödman and Kiliaridis, 2010).
- Rats are easier to handle and cheaper than larger animals (rabbits, ferrets, pigs, dogs).

"Although the findings in the rat model cannot be directly extrapolated to humans, insight into an understanding of the mechanisms that influence the interaction between muscle function and craniofacial morphology can be gained." (Ödman and Kiliaridis, 2010).

Paper I:

The advantage of using a preprogrammed custom shaped snake, which follows the whole contour of the mandible, instead of cephalometric points when studying the morphology of the lateral view of the mandible is the possibility to depict the correct shape of the mandible. This allowed us to calculate the mean size of the mandible. I consider that the shape of the mandible is easier to be detected on this type of photo than on a radiograph.

Paper II:

Photographic analysis, which was used in paper I only provides cross-sectional data and cannot be used for studying longitudinal growth changes of craniofacial morphology and therefore cephalometric analysis was preferred in Paper II, allowing registration at consecutive occasions.

Still cephalometric methods may include different types of methodological errors that can be due to a wrong positioning of the rat in the cephalostat as well as incorrect identification of cephalometric landmarks.

A disadvantage with studying the transverse dimensions by using axial cephalograms may be that they show three-dimensional structures in 2D, which may obscure the detection of other changes that may have occurred in another plane.

Paper III:

There are advantages of using microtomographic histomorphometry instead of using histological sections. For microtomographic histomorphometry there is no need to embed the mandibles in methyl methacrylate. Cutting coronal sections of embedded mandibles in a microtome instead is time consuming and it can be hard to perform at the desired position. On the other hand we had to cut the mandibles into three pieces, as the machine could not accommodate large samples. Another advantage of Micro-CT or other high-resolution 3D imaging techniques is that they directly measure bone microarchitecture without relying on stereologic models. (Bouxsein et al. 2010)
Compared to using coronal sections however, the method is an expensive method due to the cost of the machine.

Paper IV:

In earlier performed animal experimental studies histochemical methods were often used to study the change of fibre composition in the muscle (Kiliaridis et al. 1988). Histologically, muscle fibres are classified in relation to which MyHCs they contain. But it is now known that a number of MyHCs can be expressed in the same muscle fibre. Due to that we chose a method to study the MyHC 'profile' (protein and/or gene expression) of the muscle. According to Harzer et al. (2010) the presence and activity of MyHCs are the most important markers noted under adaptation and conditions of training to changed function.

RESULTS

Body weight

The different types of diet did not result in statistical significant weight differences between the groups under study. The rats in the hypofunctional and rehabilitation group had a ”normal” weight gain even though they did not receive the ordinary hard pellets.

Morphology of the lateral view of the mandible (Paper I)

The only statistical significant difference when comparing the rehabilitation group and the hypofunctional group was the inclination of the condylar base. The inclination was larger in the rehabilitation group.

In the rehabilitation group compared to the normal group the condylar process was less steep, the height of the angular process was smaller and the molar alveolar anterior and posterior heights were larger

No statistically significant differences were detected in the total area and posterior height of the corpus between the rehabilitation group and the normal group, as it was difference between the normal group and the hypofuntional group. This could indicate on a catch-up tendency in the rehabilitation group towards the normal group. (*Figure 4*)

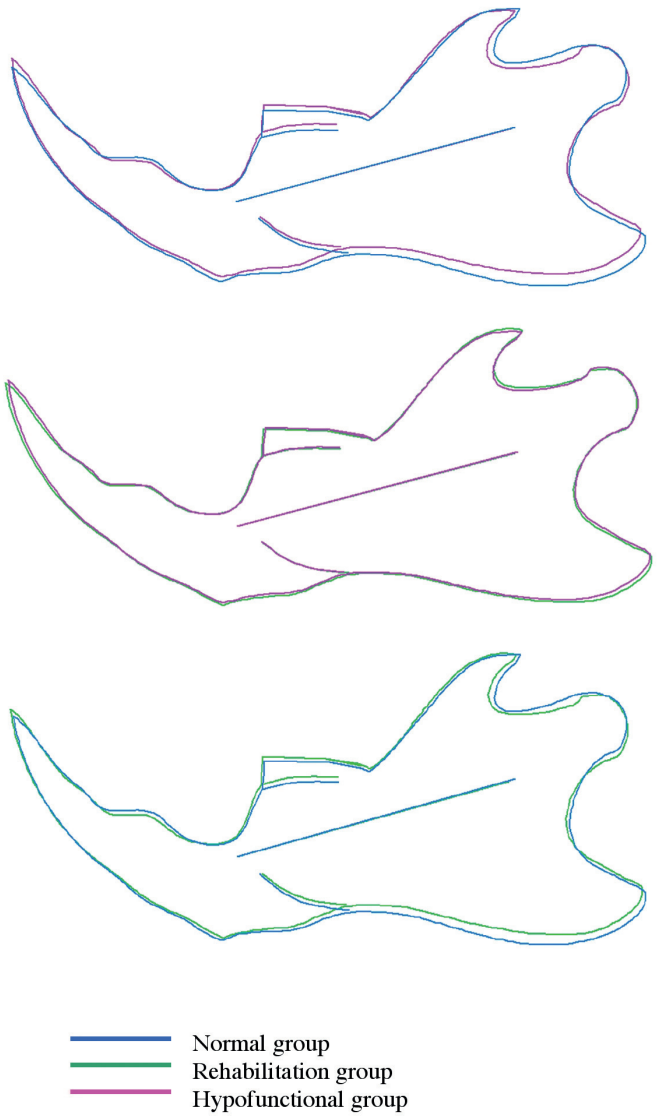


Figure 4: Superimpositions of the mean tracings on the reference line.

First: Normal group (blue line) versus Hypofunctional group (pink line).
Second: Rehabilitation group (green line) versus Hypofunctional group (pink line).
Third: Normal group (blue line) versus Rehabilitation group (green line).

(Modified from Ödman et al 2008)

Transverse skull dimensions (Paper II)

Changes in the transverse dimensions

The increase of the anterior zygomatic arch width (Z2L-Z2R) and the interzygomatic width (Z3L-Z3R) during the experimental period were larger in the rehabilitation group compared to both the normal and the hypofunctional group.

Transverse dimensions at week 21

The posterior premaxillary width (PM2L- PM2R), the anterior zygomatic width (Z1L- Z1R) and the anterior zygomatic arch width (Z2L- Z2R) were narrower in the experimental group compared to the control group. No other significant differences were detected between the groups.

Transverse dimensions at week 27

In the rehabilitation group compared to the hypofunctional group the anterior zygomatic arch width (Z2L-Z2R) was wider. There were similar trends for the interzygomatic width (Z3L-Z3R) and the intercondylar width (CDL-CDR), but they did not reach a statistical significant level.

In the rehabilitation group compared to the normal group the anterior zygomatic arch width (Z2L-Z2R), the premaxillary width (PM2L-PM2R) and the anterior zygomatic width (Z1L-Z1R) were narrower.

There were no other statistically significant differences between the rehabilitation group and the two other groups.

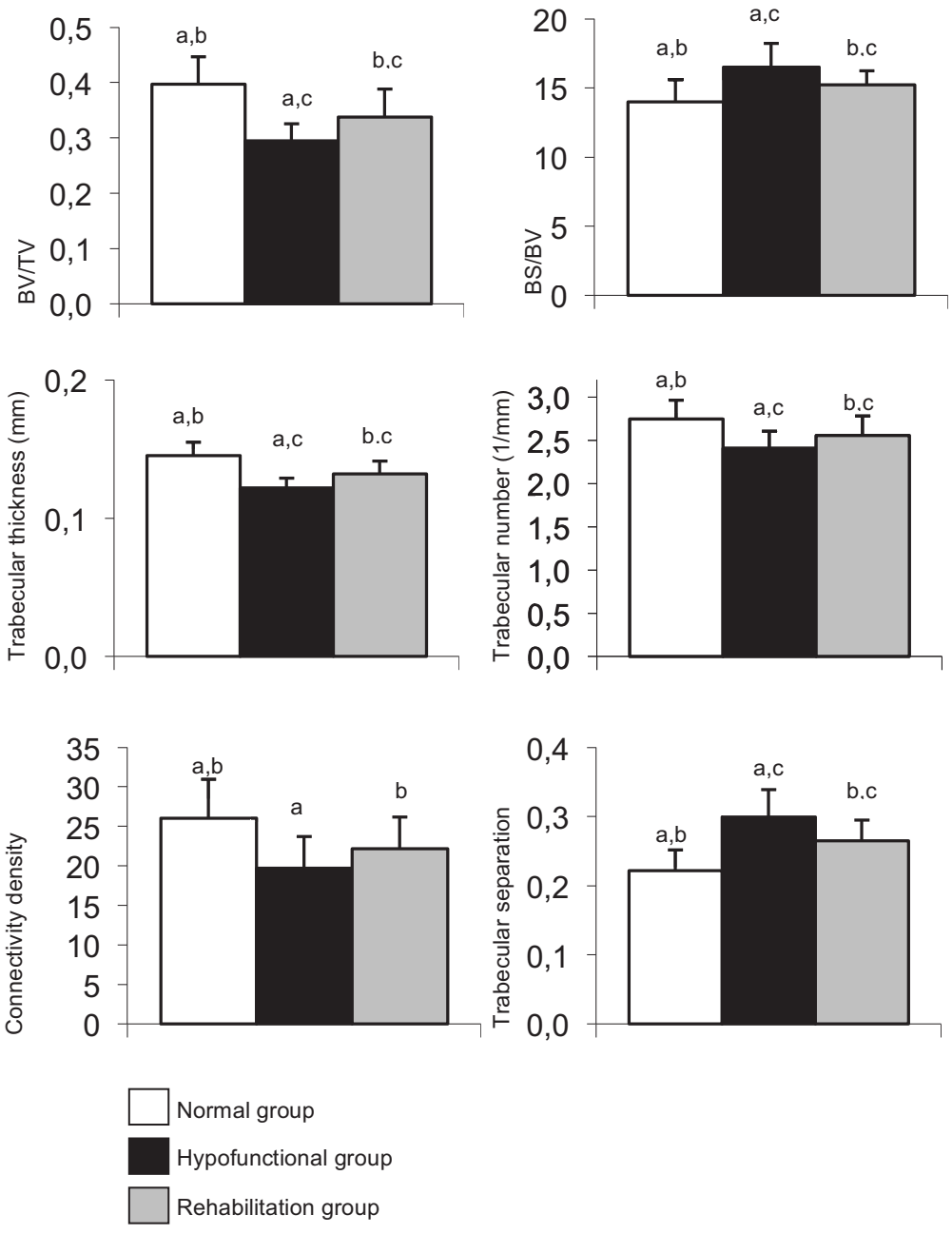
The morphology and the trabecular architecture of the mandibular alveolar bone (Paper III) *(Figure 5)*

The alveolar process was wider in the normal and rehabilitation group compared to the hypofunctional group, but shorter in height in the normal group compared to the rehabilitation and hypofunctional group.

The alveolar process trabecular bone volume fraction (BV/TV) was lower for the animals of the hypofunctional group as compared to those of the normal and the rehabilitation groups. Despite the significant improvement observed in the rehabilitation group, their BV/TV was lower in comparison to the normal group. The same relationship with the values of the rehabilitation group being in between the other groups occurred for the trabecular thickness, trabecular number, trabecular seperation.

Concerning the connectivity density it was lower in the rehabilitation group and the hypofunctional group compared to the normal group, but in this comparison between the groups there was no significant difference between the rehabilitation and hypofunctional group.

Figure 5: Micro-tomographic histomorphometric comparisons between groups. Analysis of variance was followed by post-hoc pair-wise comparison between groups. Bars with the same superscript letter are significantly different ($p < 0.05$). (BV/TV: Trabecular bone volume fraction. BS/BV: Specific bone surface) (Published by Mavropoulos et al. 2010)


BV/TV
0,5
0,4
0,3
0,2
0,1
0,0
a,b
a,c
b.c
BS/BV
20
15
10
5
0
a,b
a,c
b.c
Trabecular thickness (mm)
0,2
0,1
0,0
a,b
a,c
b.c
Trabecular number (1/mm)
3,0
2,5
2,0
1,5
1,0
0,5
0,0
a,b
a,c
b,c
Connectivity density
35
30
25
20
15
10
5
0
a,b
a
b
Trabecular separation
0,4
0,3
0,2
0,1
0,0
a,b
a,c
b.c
Normal group
Hypofunctional group
Rehabilitation group

Contractile protein gene expression in the deep masseter muscle (Paper IV)

The gene expression of MYH 1 and MYH 2 in the deep masseter muscle did not significantly vary between the three groups.

The results showed that the gene expression of MYH 3 and MYH 7 were significantly higher in the rehabilitation group compared with both the normal group and the hypofunctional group. The levels were highest in the rehabilitation group and lowest in the normal group.

DISCUSSION

This project has shown that the simulation of functional rehabilitation by retraining of the masticatory muscles led to an increase in the gene expression of MYH 3 and MYH 7 in the deep masseter muscle in adult rats. The alveolar trabecular bone architecture of the mandible did improve during the 6 weeks of retraining. However, the effects of the earlier period of 21 weeks with low masticatory demands were not completely reversed. The retraining also led to an increase in width of the mandibular alveolar process and a catch-up tendency on the lateral morphology of the mandible. The transversal dimensions of the skull increased in width on sites of origin of masticatory muscle, i.e. on the zygomatic arch, as an effect of masticatory muscular retraining.

Masticatory muscle retraining and its effect on the MYH composition in the masseter muscle

In the rehabilitation group the gene expression of MYH 7 (slow fibres) were significantly higher compared with the expression in the hypofunctional group and the normal group.

The high level of MYH 7 shows that the training by changing from soft to hard diet in the rehabilitation group affected the gene expression in the masseter muscle. The increase of slow MyHC is a mechanism of adaptation of the muscles that allows them to withstand new functional conditions (Korfage et al. 2005).

Several authors (Adams et al. 1993, Pette 2002, Barton-Davis et al. 1996) reported changes towards slower and fatigue resistant fibre types after muscular overload, which is in line with our results.

When functional demands change due to an increase of mechanical load for a healthy muscle it needs to adapt to the new circumstances. If the muscle stress increases for a long period of time, also the slow fibre types increase since they are more fatigue resistant, whereas after immobilization the IIx myosin isoforms increase (fast type) (Harzer et al. 2010).

The increased level of MYH 3 could be due to the need of creating new MyHC isoforms i.e. an adaptation to the new functional demands after retraining the masseter muscle. Oukhai et al. (2011) concluded in their preliminary study that MYH 3 could play a role in the functional adaptation of the muscles after orthognatic surgery. Their conclusion is in line with our findings where the rehabilitation group had the significantly highest level of the gene expression of the MYH 3 of the three groups under study. Another reason for the MYH 3 levels to increase could be that the embryonic isoforms may be a part of a developmental repair system (Hunt et al. 2006).

Clinical studies have shown for example increase of expression of slow MYH six months after orthognatic surgery of class II cases (Breuel et al. 2013). According to Harzer et al. (2010) the presence and activity of MyHCs are the most important markers noted under adaptation and conditions of training to changed function.

The fact that the masseter muscle was affected by the retraining shows that this experimental model, by changing the diet from soft food to hard food to increase the demands on the masticatory muscle system, is a suitable animal model that can provide us the possibility to resemble functional changes that may occur after orthognathic surgical treatment of individuals with severe malocclusions or after prosthetic rehabilitation.

Masticatory muscle retraining and its effect on the transverse dimensions of the skull and the lateral morphology of the mandible

Concerning the transverse dimensions at week 21 (start of retraining period), the posterior premaxillary width, the anterior zygomatic width and the anterior zygomatic arch width were narrower in the experimental group (fed soft diet) compared to the normal group. No other significant differences were detected between the groups.

At the end of the retraining period, those three previously mentioned cranial transverse dimensions were narrower in the rehabilitation group and the hypofunctional group compared to the control group.

In the hypofunctional group compared to the "rehabilitation" group the anterior zygomatic arch width was narrower. There were trends for similar differences for the interzygomatic width and the intercondylar width but they did not reach a statistical significant level.

As regards the posterior skull width that is probably an area that was not subjected to masticatory muscle load. We could not detect any statistically significant differences between the three groups here.

Still the effect of retraining was measurable in the "rehabilitation" group, which had the largest increases in some transversal dimensions, such as the anterior zygomatic arch width and the interzygomatic width.

Thus the increase in the transverse dimensions was most evident at sites of masticatory muscle origin on the zygomatic arch. This can be due to the need of bone remodeling adaptation to the loads resulting from the increased

functional demand on the masseter muscle.

The findings on the transverse dimensions after a prolonged period of low masticatory demands are similar to those of Katsaros et al. (2002) and of Abed et al. (2007) on growing animals. They concluded in their studies that the transverse skull dimensions were significantly negatively affected in the hypofunctional group, especially in areas under direct masticatory muscle influence, such as the sites of the masseter muscle origin. The effects of normal function they detected during the growth period were larger than those of retraining that we detected during adulthood. This could mean that a continuous modelling/remodelling still exists during adulthood, but at a lower degree than during growth. While masticatory functional demand may have a larger impact on the overall craniofacial morphology during growth, its effect seems to be limited to a local bone adaptation in adulthood.

Concerning the sagittal morphology of the mandible, the effect of retraining was only marginal compared to the animals in the hypofunctional group, but there was a catch-up tendency towards the values of the normal group.

The only statistical significant effect of retraining on the sagittal morphology of the mandible was the inclination of the condylar base. The inclination was larger in the re-trained group (rehabilitation group). The larger condylar base inclination of the rehabilitation group compared with the hypofunctional group could be the consequence of appositional changes on the posterior corpus region. This could also be a possible geometrical effect due to increased bone apposition in the region of the upper mandibular notch distal to the coronoid process, that may represent a local bone response to better withstand the increased contracting forces of the masticatory muscles and more specially the temporal muscle, which inserts in this region. The catch-up tendency in the rehabilitation group may be the reason that no statistically

significant differences were detected in the total area and in the posterior height of the corpus between the rehabilitation group and the normal group. In contrast to what was found between the normal group and the hypofunctional group. Therefore retraining in adulthood seems to have a minor modeling effect on one vertical and on the overall dimension of the mandible.

Retraining and its effect on the alveolar bone of the mandible

Low masticatory demands for a prolonged period of time resulted in an increased height, but a narrower alveolar process width compared with the alveolar process of the rats in the normal group. The retraining period in the rehabilitation group did not have a detectable impact on the increased alveolar height, but the width on the other hand normalized.

Our findings regarding the prolonged period of low masticatory demands and the effect on the alveolar bone height and width are in line with studies performed on growing animals (Bresin et al. 1994, Mavropoulos et al. 2004). However, our findings after 27 weeks are more pronounced, which may be explained by the longer period of low demands in our study compared with the mentioned studies were it was 4-6 weeks. This finding is similar to what is noticeable at different types of neuromuscular diseases with hypotonic masticatory muscles, like for example myotonic dystrophy (DM1), where the molars may continue to erupt and the alveolar bone follows (Kiliaridis and Katsaros 1989).

The fact that the increased height of the alveolar process did not normalize is not surprising as the increased height occurred during growth when the eruption of the molars occurs, and chewing forces were probably not enough

to intrude the molars and reduce the height of the alveolar process in adult animals. The increased width in the rehabilitation group is a probable sign of an adaptation of the alveolar bone to withstand the increased masticatory loads on to the molars.

Retraining and the effect on the internal structures of the mandible

"Rehabilitation" by retraining the masticatory muscle function for 6 weeks leads to an increased force load on the mandibular alveolar process. This was associated with a significant increase of the alveolar process trabecular bone volume fraction, the trabecular thickness, and trabecular number as well as a significant decrease of the trabecular separation.

Despite the significant improvement observed in the rehabilitation group, the alveolar process trabecular bone volume fraction was lower in comparison to the normal group. The same relationship occurred for the trabecular thickness, and trabecular number. The trabecular separation was however higher.

The connectivity density was not affected in the same way as the other parameters under study. No significant differences were detected between the rehabilitation group and the hypofunctional group. The connectivity density was lower than for the normal group.

Comparison with other studies on growing animals

In growing rats, a soft diet and the consequent reduction of masticatory functional demands resulted in lower bone mass and alveolar bone density in the mandible (Mavropoulos et al. 2004). This is in line with the observations

in the hypofunctional group in our study.

In the mentioned article part of the rats under study had fixed upper bite block. This led to a continuous force of the mandibular alveolar process. The force in turn gave a significant increase of the cortical bone thickness. In adult rats it is known that exercise in form of treadmill increases cancellous BV/TV of the tibia (Yeh et al. 1993) and increases the mineral and bone formation rate in the tibia (Chen et al. 1994). Our results on the alveolar process confirm their results.

Why these results:

According to Iwamoto et al. (2005) the effect on the cortical bone of the tibia is due to the stimulation of the periosteal bone formation and a suppression of the endocortical bone resorption. At adulthood there is a decreased bone formation compared with growing individuals (Gardiener et al. 2018), which may be an explanation to why the “rehabilitation” group did not reach the same levels as the normal group concerning the trabecular parameters. If the experimental period had been extended for a longer period of time the “rehabilitation” group might have reached the levels of the normal group.

A prolonged period of retraining might also have affected the morphology of the mandible and the transverse dimensions of the skull in a more extended way, as the bone formation is slower in adulthood than during the growth period. During growth instead, a period of 4 weeks with different functional masticatory demands can be enough to produce obvious differences in mandibular morphology (Kiliaridis et al. 1985, Bresin et al. 1994, Mavropouls et al. 2004).

Clinical implications of our findings

Although the findings from an experimental study cannot be directly extrapolated to humans, they suggest that craniofacial skeleton seems to have some potential for bone modelling adaptation in response to improved masticatory function/increased loads even in adulthood. In can be speculated that an improvement of the masticatory muscle function after surgical correction of severe class II, III and open bite malocclusions or extensive prosthetic rehabilitation could affect certain sites of the skull and local internal bone structures. Those functional changes may have effect even in adults, although at a slower pace, since it is known that "renewed modeling" in the adult skeleton can occur in cases where the mechanical loading has been altered significantly (Robling et al. 2006). We can further speculate that the skeletal response on the craniofacial morphology due to improved masticatory muscle function could occur in adulthood and may help improve the stability of the result of orthognathic surgery in adult individuals. Clinical trials need to be performed to answer this question. They should be done at least one or few years after orthognatic surgery or after dental implant placement in order to see the effects on long term.

In line with our findings and speculations, some authors have even further suggested that training of the masticatory musculature could help to diminish the risk for relapse after orthognathic surgery (Gedrange et al. 2006). Whether that is the case or not, it remains to be demonstrated.

CONCLUSION

The increased functional demands from retraining in adults seem to influence the craniofacial morphology at areas under direct influence of the masticatory muscles or increased loading.

- There were only minor effects on the morphology of the lateral view of the mandible the observed catch-up tendency might suggest that a longer retraining period might have a more substantial effect on the mandibular morphology.
- The retraining of masticatory muscles in adult rats leads to an increase of some transverse craniofacial dimensions, a possible result of bone remodeling to resist loads on the areas of origin/insertion of masticatory muscles.
- The increased forces that the alveolar process of the mandible was exposed to due to the increased functional demands affected the alveolar process width it became slightly wider, but not the height.
- Alveolar trabecular bone architecture did improve after functional retraining although the effects of hypofunction were not completely reversed. A longer retraining period might have a more substantial effect on the trabecular bone.
- Retraining of the deep masseter muscle made the slow contractile gene expression isoform (MYH 7) levels to increase, an adaptation to the increased mechanical load. The increased level of embryonic gene expression isoform (MYH 3) can be due to the need for the creation of new MyHC isoforms.

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