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Ultrastructural abnormalities of muscle spindles in the rat masseter muscle with malocclusion-induced damage

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Summary. Human temporomandibular disorders due to disturbed occlusal mechanics are characterized by sensory, motor and autonomic symptoms, possibly related to muscle overwork and fatigue. Our previous study in rats with experimentally-induced malocclusion due to unilateral molar cusp amputation showed that the ipsilateral masseter muscles undergo morphological and biochemical changes consistent with muscle hypercontraction and ischemia. In the present study, the masseter muscle spindles of the same malocclusion-bearing rats were examined by electron microscopy. Sham-operated rats were used as controls. In the treated rats, clear-cut alterations of the muscle spindles were observed 26 days after surgery, when the extrafusal muscle showed the more severe damage. The fusal alterations affected predominantly capsular cells, intrafusal muscle fibers and sensory nerve endings. These results suggest that in the malocclusion-bearing rats, an abnormal reflex regulation of the motor activity of the masticatory muscles may take place. They also allow us to hypothesize that muscle spindle alterations might be involved in the pathogenesis of human temporomandibular disorders.

Key words: Muscle spindle, Masseter muscle, Malocclusion, Ultrastructure

Introduction

Increasing evidence has been accumulating that pain and dysfunctional syndromes of the masticatory apparatus, usually referred to as temporomandibular disorders (TMDs), may take place in subjects with disturbed occlusal mechanics (Dawson, 1974; Kirveskari, 1978). Yet despite many years of study, the pathophysiology of TMDs has remained elusive. It has been hypothesized that malocclusion may influence the functional performance of the masticatory muscles, thus resulting in muscular overwork and fatigue (reviewed in Simons et al., 1999). Among the possible factors involved in muscle dysfunction, an abnormal behavior of muscle spindles has been proposed (Hubbard and Berkoff, 1993; Hubbard, 1996). Muscle spindles are sense organs located in the skeletal muscles where they serve as mechanoreceptors sensitive to muscle length and its changes. Their principal function is the unconscious control of muscle tone and motor activity (Boyd and Smith, 1984). The idea that muscle spindles might be involved in the functional derangement of the masticatory muscles of malocclusion-bearing subjects also comes from experimental studies in cat limb muscles, in which long-lasting contractions and ischemia have been shown to cause changes in the activity of muscle spindles consistent with an increase in the fusimotor discharge rate (Ljubisavljevic et al., 1992).

In a recent study, we set up a rat model in which the induction of malocclusion caused morphological and biochemical changes of the masseter muscle consistent with muscle hypercontraction and ischemia (Bani et al., 1999). The present study aims at investigating whether structural changes may take place in the muscle spindles of the rat masseter with malocclusion-induced damage.

Materials and methods

Twenty male albino rats, Wistar strain, weighing 250-300 g (Harlan Nossan, Correzzana, Italy) were used. They were quarantined for 7 days at 22-24 °C on a 12-h light, 12-h dark cycle before use. Standard hard pelleted chow (Harlan Nossan) and water were available ad *libitum*. The experimental protocol was designed in compliance with the principles of laboratory animal care (NIH publication No. 86-23, revised 1985) and the recommendations of the European Economic Community (86/609/CEE) for the care and use of laboratory animals. Moreover, this was approved by the animal care committee of the University of Florence (Italy). The rats were randomly distributed in 2 groups of 10 animals each and treated as described below.

The rats of the first group were anesthesized by i.p. injection of ketamine (Parke Davis, Milan, Italy; 150

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mg/kg body weight). Jaws were gently opened using silk threads passed around the lower and upper incisors. Mechanical reduction of the cusp height was carried out to bring the teeth out of occlusion. Briefly, the superior and inferior molar cusps of the left hemiarcades were ground with ball-shaped diamond bur, 2 mm in diameter, under flushing with cold saline to prevent overheating. Care was taken not to cause pulp exposure or damagement. The trauma during jaw opening and the duration of the surgical treatment (about 2 min per rat) were kept to a minimum to avoid stretch-induced damage of the masticatory muscles. The rats of the second group underwent the same treatment except for molar cusp amputation and were used as controls.

To exclude that the possible differences between the control and the experimental rats could be ascribed to different alimentary behaviour caused by dentin exposure and related pain and avoidance patterns, the rats of both groups were placed into individual cages with a predetermined amount of chow (100 g) and the daily food consumption was measured for the entire duration of the experiment. Food consumption was similar in both the groups, apart from the first 48 h, in which the rats with amputated molar cusps ate substantially less food than the controls (Bani et al., 1999). After 14 days, 5 rats from each group were killed by prolonged ethyl ether anesthesia. Upon sacrifice, the masseter muscles ipsilateral to the ground hemiarcades were quickly excised. Tissue fragments were cut and processed for electron microscopy, as described below. The remaining rats, 5 with molar amputation and 5 sham-operated controls, were killed 26 days after intervention and underwent the same sampling procedure as above. We chose to investigate the masseter muscles ipsilateral to the amputated molars because in a previous study on the same rats used here we have shown that substantial muscle tissue changes following unilateral malocclusion are almost exclusively localized in the ipsilateral masseter (Bani et al., 1999).

For electron microscopy, the tissue fragments were cut in small pieces, fixed by immersion in cold 4% glutaraldehyde in 0.1M sodium cacodylate buffer, pH 7.4, for 3 h at room temperature, and postfixed in 1% osmium tetroxide in 0.1M phosphate buffer, pH 7.4, for 1 h at 4 °C. They were then dehydrated in graded acetone, passed through propylene-oxide and embedded in Epon 812 (Fluka, Buchs, Switzerland). Semi-thin sections, 2 μm thick, were stained with toluidine blue-sodium tetraborate and observed at a light microscope to localize muscle spindles. Ultra-thin sections were then cut, stained with uranyl acetate and alkaline bismuth subnitrate and viewed under a JEM 1010 electron microscope (Jeol, Tokyo, Japan) at 80 kV. In each rat, at least 2 muscle spindles, each coming from a different tissue fragment, were examined. In this way, a minimum of 10 muscle spindles was analyzed in each experimental group. Observations were preferentially carried out in the equatorial and juxtaequatorial regions, where sensory nerve endings are concentrated. In some cases, the polar regions where motor nerve endings are located were also examined.

Results

Control rats

In the control rats, the muscle spindles had a normal appearance. They were usually composed of a thin, multilayered capsule surrounding intrafusal muscle fibers and associated sensory and fusimotor nerve endings.

The capsule was made up of an outer capsule enclosing a periaxial space filled with electron-lucent matrix, and a thin inner capsule adjacent to intrafusal muscle fibers and sensory nerve endings. The outer capsule was composed of concentrically arranged, flattened perineural cells joined by tight junctions, surrounded by a basal lamina and containing many pinocytotic vesicles (Fig. 1A). Thin collagen fibrils and small blood capillaries were interposed between the cell layers. The inner capsule was composed of a few perineural cells poor in pinocytotic vesicles and other cells featuring quiescent fibroblasts with long, slender cytoplasmic processes and no basal lamina (Fig. 1B). Beneath the inner capsule, thin collagen fibers and large elastic fibers were seen.

The intrafusal muscle fibers had the well-known ultrastructural features described in the literature (Boyd and Smith, 1984). Usually, there were two nuclear bag fibers and two-to-four nuclear chain fibers, which could be distinguished on the basis of the nuclear distribution, the characteristics of myofibril cross banding, the amount of mitochondria and of sarcoplasmic reticulum, and the relationships with the surrounding elastic fibers. (Fig. 2A,B). Satellite cells were seldom encountered apposed to'intrafusal muscle fibers.

Sensory nerve endings, closely adherent to the intrafusal muscle fibers, showed densely packed mitochondria and small-sized coated and uncoated vesicles (Fig. 2B,C). In distinct areas of the two apposed membranes, intermediate junctions could also be seen (Fig. 2C). On the side opposite to the muscle fibers, the sensory terminals were lined by a basal lamina continuous with that of the muscle fibers. No Schwann cells covered the terminals.

Fusimotor nerve endings were located at the poles of the intrafusal muscle fibers and contained many synaptic vesicles and mitochondria. They were always covered by Schwann cells.

As a rule for mammals (Rokx et al., 1984; Eriksson and Thornell, 1987; Rowlerson et al., 1988; Bredman et al., 1991; Sciote and Rowlerson, 1998), we found muscle spindles in tissue areas rich in type I (also called red, or slow) muscle fibers, known for having a predominantly oxidative metabolism (Goldspink, 1983). In these areas, the extrafusal muscle fibers as well as the capillary endothelia had a normal appearance. Of note, capillaries were located within grooves at the surface of

muscle fibers and had open lumina.

Rats with molar amputation

In the rats with molar amputation since 14 days no substantial changes could be observed in the electron microscopic appearance of the different muscle spindle components, apart from occasional mitochondria1 alterations in the intrafusal muscle fibers. These alterations consisted of zig-zag-shaped, whorled or

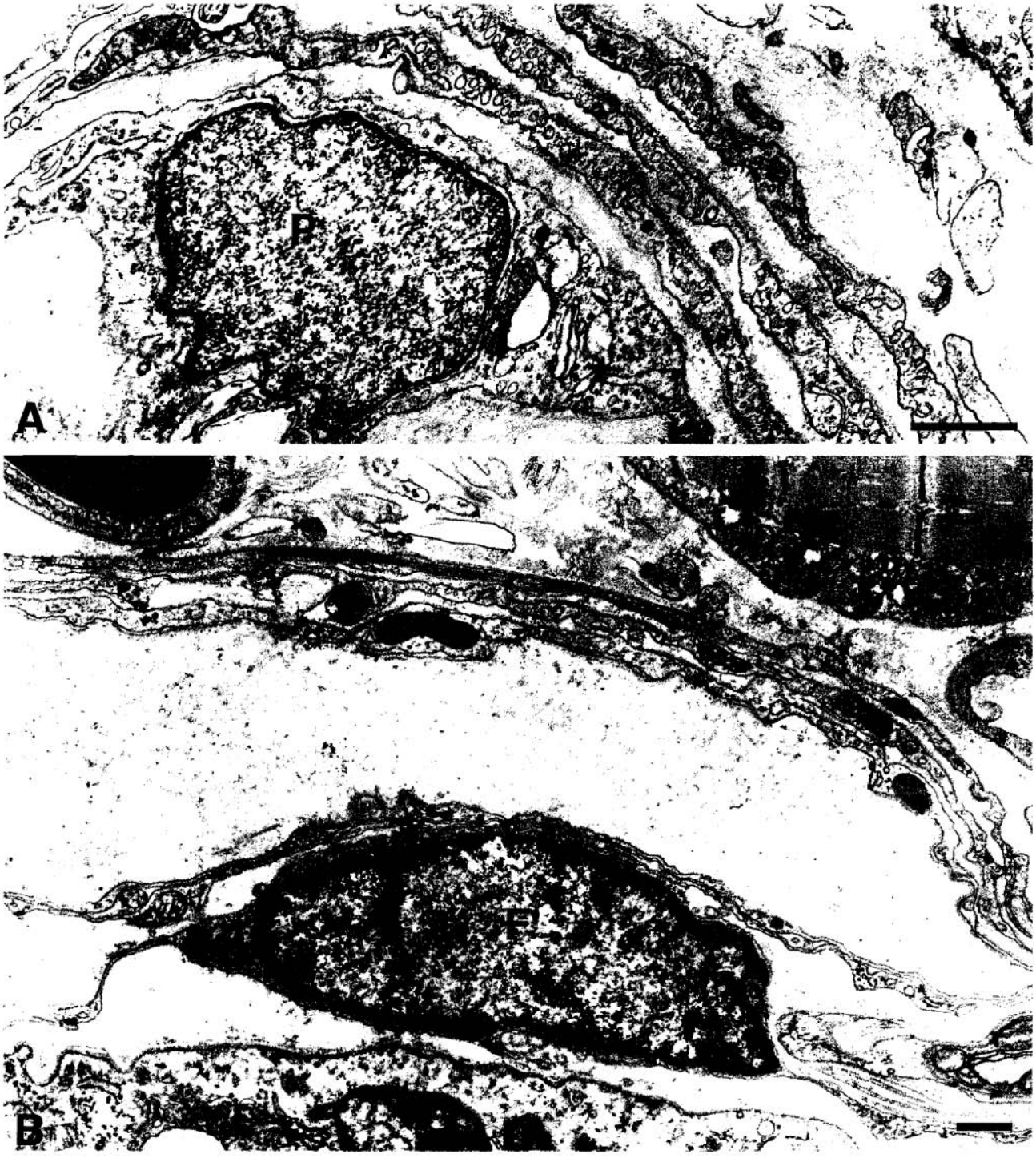

Fig. 1. Control rats: muscle spindles. **A.** The outer capsule is composed of multiple layers of flattened perineural cells (P), provided with numerous pinocytotic vesicles and a continuous basal lamina. x 20,000. B. The inner capsule shows few perineural cells poor in pinocytotic vesicles and a quiescent fibroblast (F) with long and slender cytoplasmic processes. The periaxial space is filled with electron-lucent material. x 9,000. Bars: 1 µm.

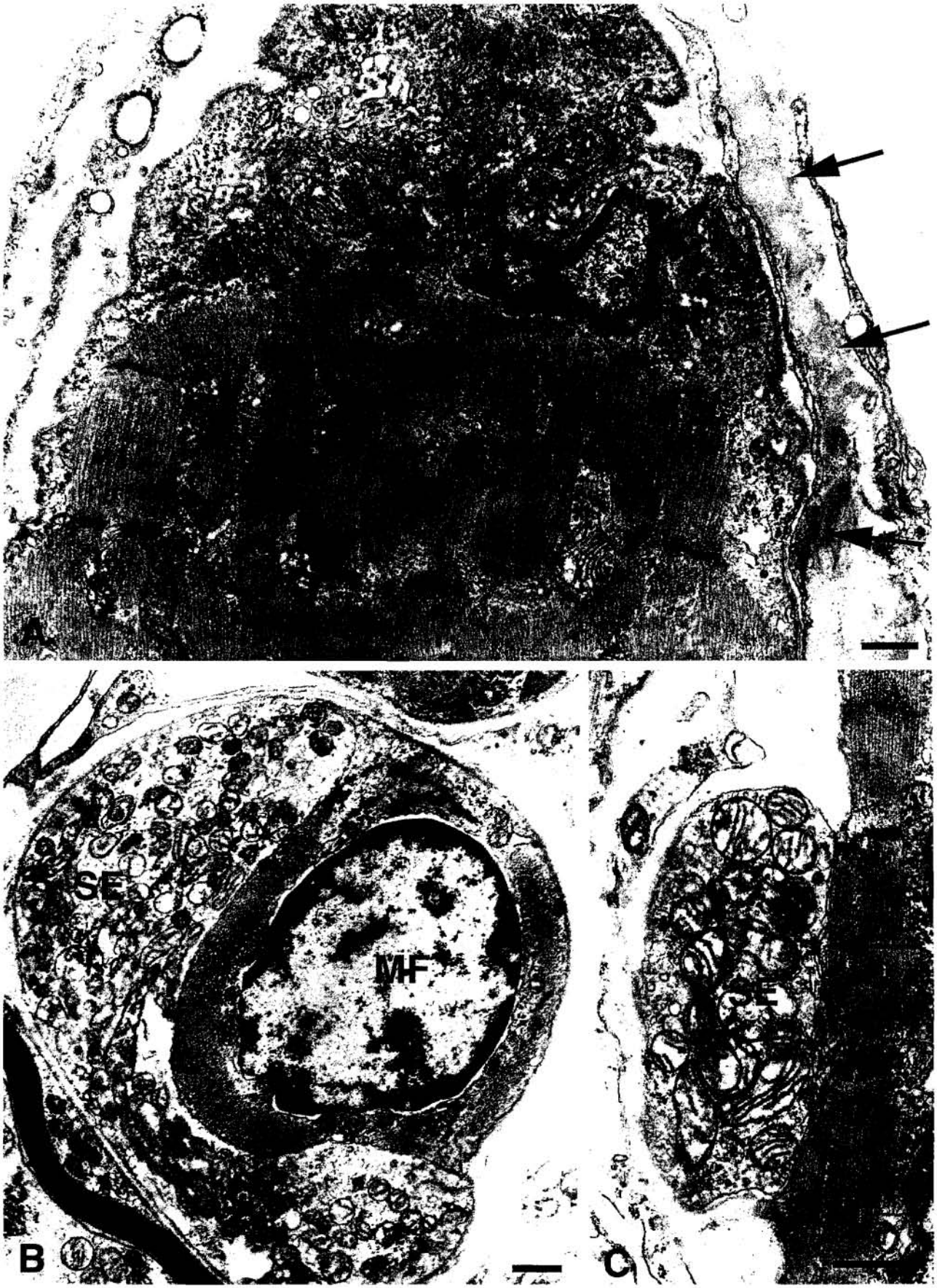
SE
MF
B
C

Fig. **2.** Control rats: muscle spindles. **A.** A nuclear bag muscle fiber cut in the juxtaequatorial region shows normal ultrastructural features. Arrows point at elastic fibers. x 18,000. **B.** A nuclear chain muscle fiber (MF) in transverse section at the equatorial region shows a very small diameter and a thin rim of myofibrils around a nucleus. A sensory nerve ending (SE) filled with mitochondria can also be seen apposed to the muscle fiber. x 12,000. **C.** A sensory nerve ending (SE) apposed to a nuclear chain intrafusal muscle fiber shows numerous mitochondria and a basal lamina continuous with that of the muscle fiber. Intermediate-like junctions can be seen between the two apposed membranes (arrows). x 25,000. Bars: 0.5 μm.

disrupted cristae, wall thickening due to close apposition between the inner and the outer membranes, and clearing of the matrix (Fig. 3A-C). Of note, the muscle tissue areas surrounding the spindles showed constricted capillaries and many extrafusal type I muscle fibers with swollen mitochondria.

In the rats with molar amputation since 26 days, clear-cut ultrastructural abnormalities were found. These abnormalities ranged from slight mitochondrial changes of intrafusal muscle fibers like those observed at 14 days to severe alterations involving all the fusal components. In this latter case, the outer capsule showed perineural cells with mitochondrial swelling, clearing of the cytoplasmic matrix, and strong reduction or even disappearance of pinocytotic vesicles (Fig. 4A). The inner capsule contained fibroblasts with enlarged Golgi apparatus and several lysosomes (Fig. 4B). The intrafusal muscle fibers showed swollen mitochondria, edematous cytoplasmic matrix, and large lysosomes (Figs. 4B, 5A-C). The sensory nerve endings also showed prominent changes, i.e. swollen mitochondria (Fig. 5C), elongated mitochondria with paracristalline inclusions (Fig. 6A,B), and presence of lysosomes and autophagic vacuoles (Fig. 6C). When examining the polar regions of muscle spindles, the fusimotor nerve endings encountered usually showed normal features, apart from sparse mitochondrial swelling of moderate degree. Extrafusal muscle fibers in the vicinity of the more severely damaged spindles often showed signs of injury, ranging from marked mitochondrial swelling to myofibril contracture or even disarrangement of the contractile apparatus. Blood capillaries were very constricted, with their lumina often reduced to a narrow cleft.

Discussion

The present findings show that ultrastructural changes take place in the muscle spindles of the rat masseter muscle affected by malocclusion-induced

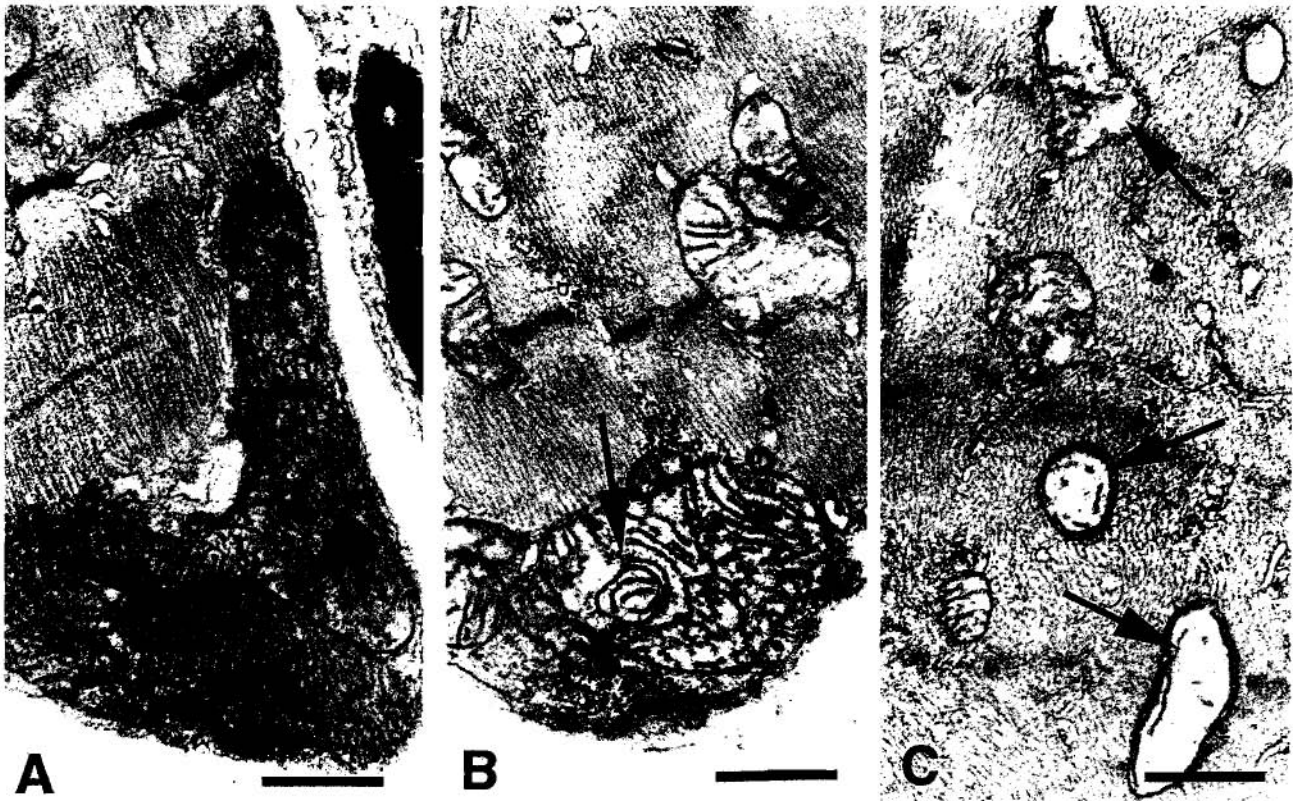

Fig. 3. Rats with molar amputation since 14 days: muscle spindles. Different kinds of mitochondrial abnormalities can be seen in the muscle fibers: **A)** zig-zag cristae (arrow) and membrane thickening (arrowheads), B) whorled cristae (arrow), **C)** disrupted cristae and clearing of the matrix (arrows). x 25,000. Bars: 0.5 pm.

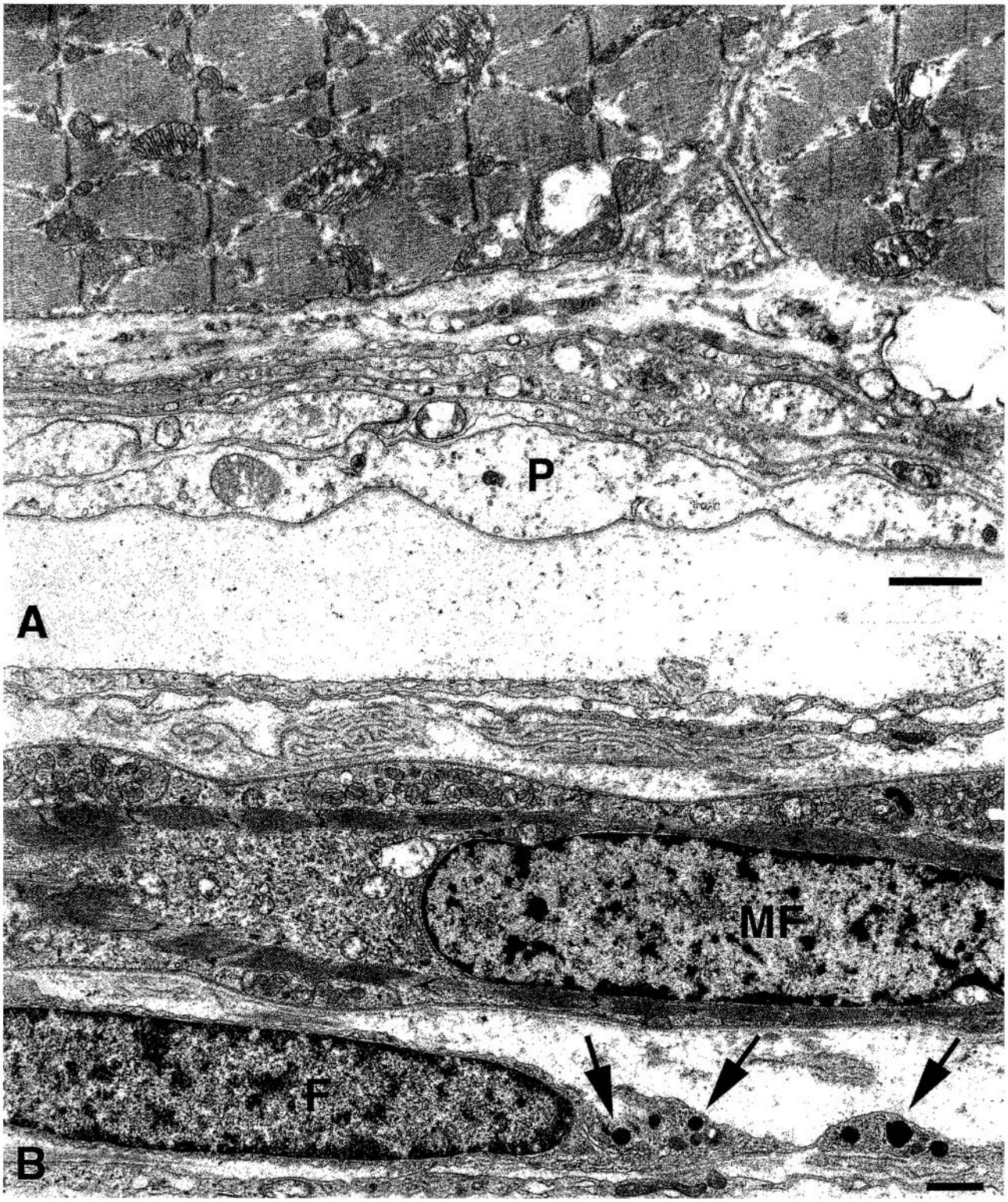

Fig. 4. Rats with molar amputation since 26 days: muscle spindles. **A.** Perineural cells (P) of the outer capsule show cytoplasmic edema, mitochondrial swelling and strong reduction of pinocytotic vesicles. x 15,000. B. A fibroblast (F) of the inner capsule shows numerous lysosomes in its cytoplasm (arrows). A nuclear chain muscle fiber (MF) with swollen mitochondria can also be seen. x 7,500. Bars: 1 µm.

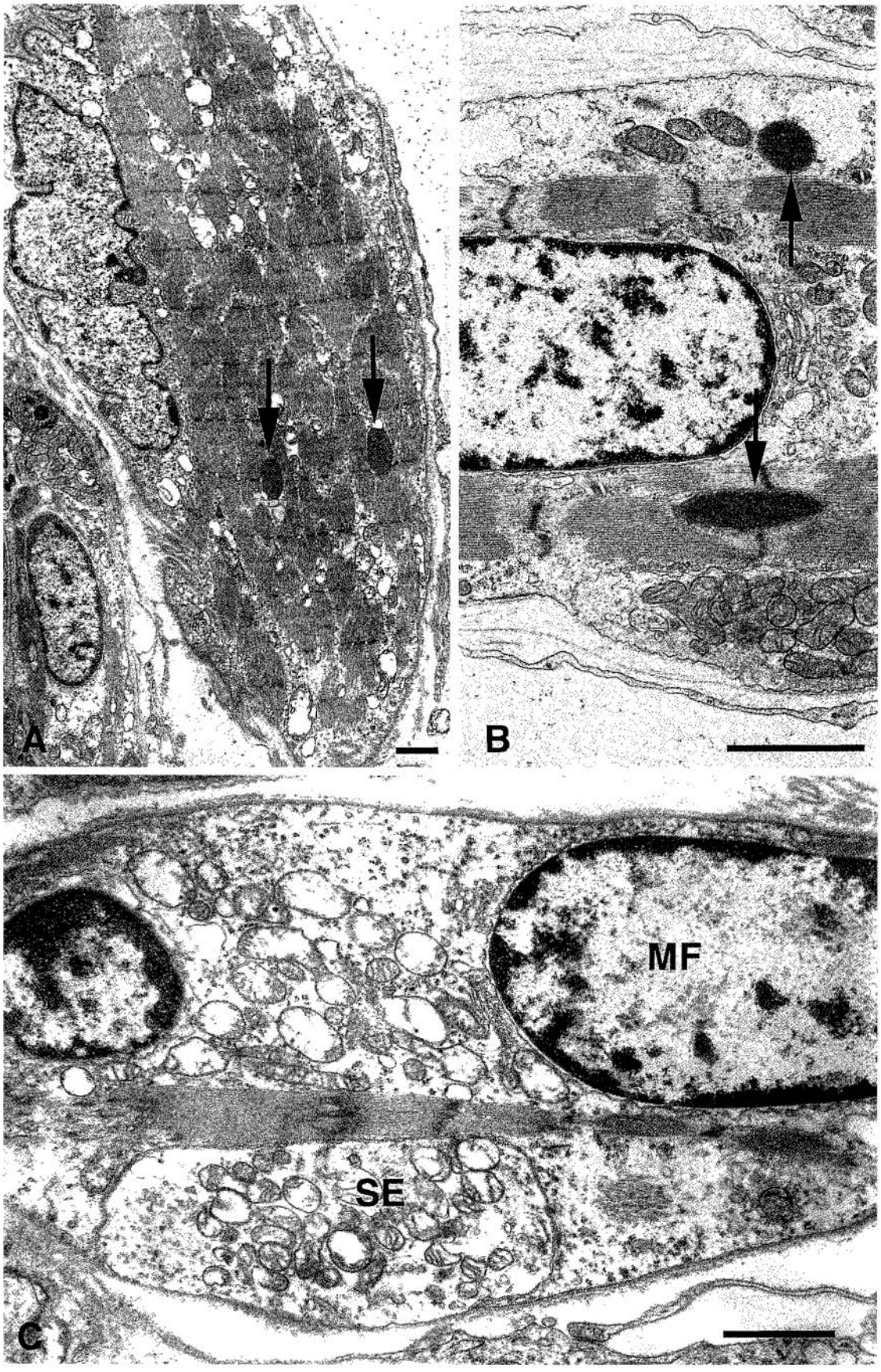

Fig. *5.* Rats with molar amputation since 26 days: muscle spindles. **A.** A nuclear bag muscle fiber shows two large membrane-bound bodies featuring lysosomes (arrows). x 7,500. **B.** A nuclear chain muscle fiber shows two lysosomes (arrows). x 20,000. ***C.*** A nuclear chain muscle fiber (MF) shows edematous cytoplasmic matrix and swollen mitochondria. Similar abnormalities can also be seen in the apposed sensory nerve ending (SE). x 18,000. Bars: ▮ µm.

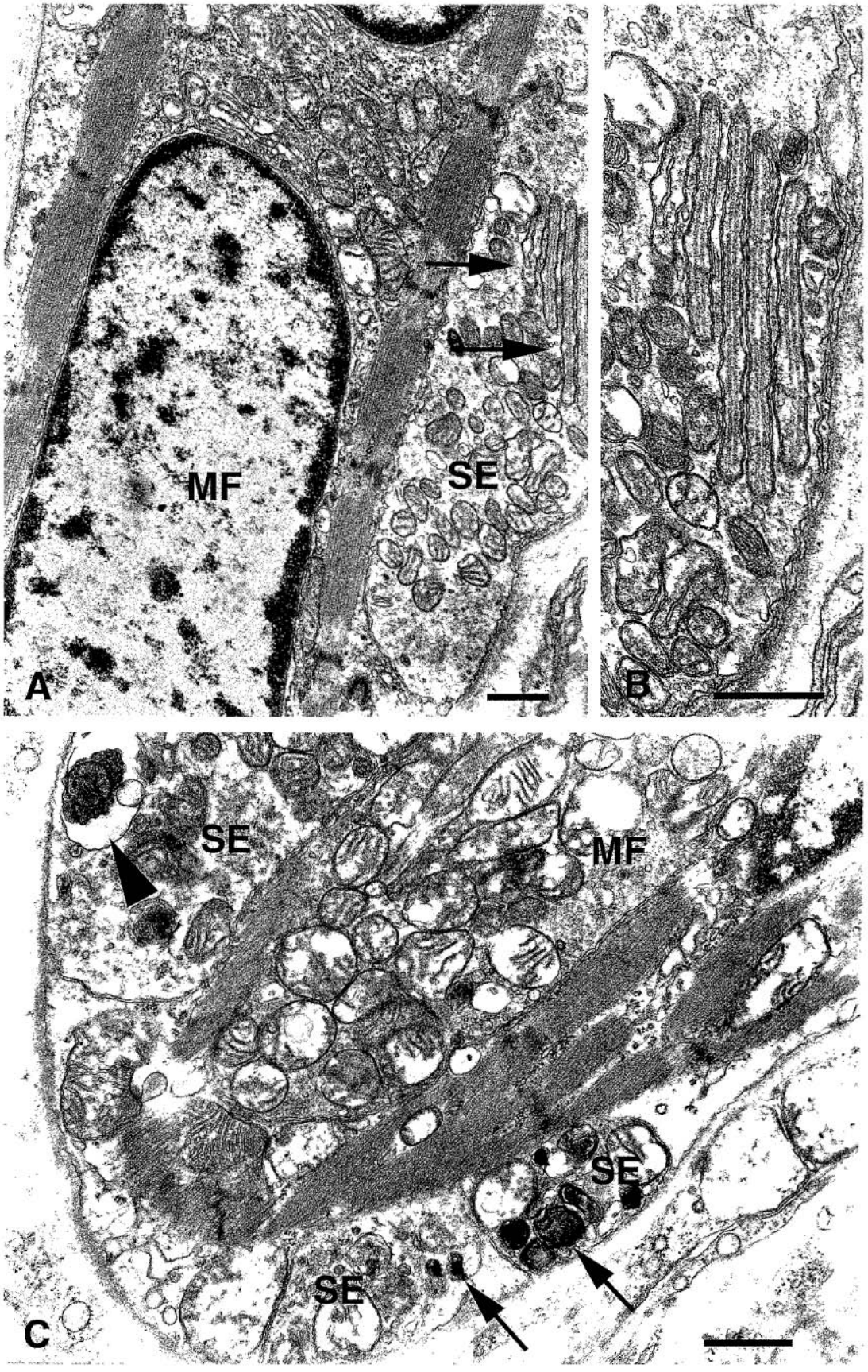

Fig. *6.* Rats with molar amputation since 26 days: muscle spindles. **A.** A sensory nerve ending (SE) apposed to a nuclear chain muscle fiber (MF) shows abnormal, elongated mitochondria with paracristalline inclusions (arrows). x 20,000. B. Higher magnification of the former figure showing in detail the paracristalline inclusions in the matrix of aberrant mitochondria. x 37,500. **C.** Sensory nerve endings (SE) containing lysosomes (arrows) and an autophagic vacuole (arrowhead) apposed to a muscle fiber (MF) with swollen mitochondria. x 25,000. Bars: 0.5 µm.

damage. At day 14 from molar cusp amputation, these changes consisted of scattered mitochondrial abnormalities of the intrafusal muscle fibers similar to those described in tissues with altered mitochondrial metabolism (Luft et al., 1962; Ernster and Luft, 1963; Gustaffson et al., 1965; Knoll and Brdiczka, 1983), or in hypoxic muscle tissues (Karpati et al., 1974). At day 26 from molar cusp amputation, muscle spindle abnormalities were more severe than in the short term experiments. These abnormalities affected not only the intrafusal muscle fibers, but also the capsular cells and the sensory nerve endings. Besides diffuse mitochondrial swelling, lysosomes appeared in the intrafusal muscle fibers, similar to those described in the advanced stages of mouse muscular dystrophy (Ovalle and Dow, 1986) and in the spindles of rat soleus muscles subjected to tenotomy (Matsumoto and Baker, 1987). The marked changes of the perineural cells of the outer capsule suggest that the damaged capsule may have lost its function of barrier towards the incursion of foreign molecules from the extrafusal interstitium, thus causing an alteration of the microenvironment in which sensory nerve endings operate (Kennedy and Yoon, 1979; Dow et al., 1980). This view is further strengthened by the occurrence of cells of the inner capsule showing cytological signs of activation of the lysosomal apparatus, in keeping with their supposed role as scavengers of foreign molecules in the periaxial space (Kennedy and Yoon, 1979; Dow et al., 1980).

Our data show that sensory nerve endings undergo clear-cut alterations. Mitochondria are markedly swollen and sometimes show paracristalline inclusions, reminiscent of those observed in muscles of patients with different kinds of myopathy (reviewed in Scochet and Lampert, 1978) as well as in hepatic cells undergoing ischemia (Swenson et al., 1967). These changes have been associated with deficient respiratory control (Karpati et al., 1973). Moreover, lysosomes and autophagic bodies are a frequent finding, thus suggesting that demolition of damaged organelles and other cytoplasmic components has occurred in the nerve endings.

It is likely that the observed changes of the muscle spindles are related to ischemia, as can be deduced by the marked constriction of blood capillaries and the occurrence of muscle fiber alterations characteristic of ischemia in the muscle tissue surrounding the spindles. A more detailed description of the changes of the extrafusal muscle tissue has been reported in a previous investigation carried out in the same animals (Bani et al., 1999).

The results from our study may have physiological implications, since they suggest that, along with the appearance of morphological abnormalities, the function of muscle spindles may also be affected. Based on the mere ultrastructural data, we cannot establish which lund of functional alteration might take place in the damaged muscle spindles. However, previous observations have shown that an increased fusimotor discharge rate occurs in cat limb muscles subjected to long-lasting fatigue and ischemia (Ljubisavljevic et al., 1992). In turn, increased fusimotor activity could enhance sensitivity of muscle spindle sensory endings, as in fact observed in fatigued muscles (Nelson and Hutton, 1985), thus causing higher than normal motor output. An increased motor activity of the masseter muscle may also depend upon an abnormal behaviour of fusal Ia afferents, which could cause hyperexcitability of the masseter a motoneurons by means of their central connections with these cells (Fritz et al., 1989). One could speculate that similar conditions might also occur in the spindles of the masseter muscles of the malocclusion-bearing rats studied here, which are subjected to functional overload and ischemia. An excess muscle tone possibly due to dysfunction of muscle spindle afferents might contribute to the masseter hypercontraction observed by us in the extrafusal muscle fibers of the malocclusion-bearing rats (Bani et al., 1999).

Caution is needed when transferring the conclusions drawn from experimental animal models to humans. Nonetheless, it is well known that muscle spindles in the human jaw muscles play a crucial role in the perception of the spatial location of the mandible and the interdental dimension (Broekhuijsen and van Willigen, 1983; Morimoto, 1983), as well as in the adaptation of the masticatory apparatus to new requirements coming from changes in jaw relationships due to occlusal alterations (Eriksson and Thornell, 1987). Our hypothesis that malocclusion may lead to muscle spindle dysfunction and excess motor activity of the masseter muscle might help to explain the occurrence of a local twitch response upon mechanical stimulation of the jaw muscles in patients suffering for TMDs (Bergamini and Prayer-Galletti, 1992; Simons et al., 1999), as well as the frequent association of disturbed occlusal mechanics and bruxism (Laskin, 1969; Trenouth, 1979).

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