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# MORPHOLOGICAL EVIDENCE OF NEUROMUSCULAR JUNCTION REORGANIZATION IN RAT OBLIQUE ABDOMINAL MUSCLES

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## ABSTRACT

The motor endplates are dynamic structures that present a high degree of plasticity which does not stop with the cessation of development, but lasts throughout life. The present study describes the ultrastructural aspects that characterize this junction renewal process in the oblique abdominal muscles of aged rats (18-24 months). About 50% of the motor endplates studied presented reorganization characteristics such as shallow primary clefts without an axonal terminal, free junctional folds, axon terminals with few synaptic vesicles and presenting pleomorphic structures, large junctional folds containing collagen, and cytoplasmic projections of Schwann cells penetrating the primary synaptic cleft. These aspects are similar to those previously described in adult rats during retraction and degeneration of the axon terminal. Although less frequent, further evidence included the presence of small nerve terminals rich in vesicles, covered by a common Schwann cell and associated with closely packed junctional folds. This last characteristic was associated with nerve sprouting and occupation of the synaptic cleft with new nerve endings. The results of this study are discussed in view of the pertinent literature and we conclude that the plasticity phenomenon of the motor endplate is present throughout life and is more frequent and intense in old animals.

**Key words:** Aging, neuromuscular junction, oblique muscles, plasticity, rat

## INTRODUCTION

Motoneurons show collateral and terminal sprouting in response to several events such as total or partial muscle denervation [2,5,7,20,24], nerve crush, or drugs that block neuromuscular transmission [25,39]. Nerve terminals at the neuromuscular junction (NMJ) have been shown to sprout or retract during development [3,22]. Morphological data indicate that terminal sprouting and retraction of motor nerves occur under normal conditions [1,4,6,8-10,15,26,27,33-37]. Using lipophilic markers, Balice-Gordon *et al.* [3] showed that axon terminal retraction occurs in a gradual and slow manner. These authors also suggested that acetylcholine receptors are removed before axon retraction. Although these changes occur throughout life, some important modifications are observed during senescence [6,11,12,14,16-19,28-32]. These studies revealed an increased myoneural junction area and length [36], and an increase in total preterminal axon length in older animals [3,12,28,39]. Wernig and Herrera [37] reported that after denervation the new synaptic contact occurs either inside or around the primitive NMJ.

Evidence for the reorganization of the myoneural junctions in normal muscles includes empty primary synaptic clefts, sarcolemmal junctional folds without axonal contact, shallow synaptic clefts exhibiting few and sparse junctional folds containing collagen fibrils, small nerve terminals in contact with an undifferentiated postsynaptic membrane, and pleomorphic nuclei and cytoplasmic processes of the Schwann cell penetrating between the axon terminal and the sarcolemma [8].

The present study describes morphological evidence obtained by transmission electron microscopy of the reorganization of myoneural junctions in oblique abdominal muscles of aged albino rats (18-24 months).

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Oblique abdominal muscles were chosen because of their continuous action during abdominal respiration in the rat. The morphological data shown here may contribute to future investigations about NMJ plasticity.

## MATERIAL AND METHODS

The study was conducted on four male Wistar rats aged 18 to 24 months. All procedures were performed in accordance with the Ethics Committee of the Instituto de Biociências, UNESP, Botucatu (SP). The animals were anesthetized by ether inhalation, followed by the intraperitoneal injection of 35 mg/g sodium pentobarbital. The oblique abdominal muscles from both sides were dissected, removed, carefully stretched and clamped onto plastic frames to avoid retraction during processing, and immersed in Karnovsky fixative.

The myoneural junctions were located as proposed by Cardasis and Padykula [9]. Muscle regions containing NMJs were removed, processed for transmission electron microscopy, and examined and photographed under a Philips EM 100 electron microscope.

## RESULTS

In a pool of specimens from eight oblique muscles, 34 myoneural junctions were analyzed by transmission electron microscopy and 16 of them presented clearly visible morphological signs of sprouting and retraction of the nerve terminals and changes in the accompanying juxtajunctional sarcolemma. The morphologically altered myoneural junctions also presented some empty synaptic clefts (Figs. 1 and 2) side by side with others of apparently normal morphology. Figure 1 shows axon terminals with a normal appearance as well as others close to disperse and broad junctional folds. The two nerve terminals on the left show morphological characteristics of normal axon terminals: the junctional folds are closely packed, the nerve terminals contain plenty of synaptic vesicles and are facing the sarcolemmal folds, and the Schwann cell processes are covering the nerve terminals over the synapse, without penetrating the synaptic cleft. In the central region of the same NMJ, one nerve terminal is detaching from the muscle fiber surface. The most striking and frequent aspect was the extensive junctional fold area not facing the corresponding axon terminal (Fig. 3). In this electron photomicrograph all nerve terminals present signs of atrophy and different degrees of abnormalities. Some nerve terminals are covered by Schwann cell processes which partially penetrate into the synaptic clefts and separate them from the junctional folds (Figs. 4 and 5).

The basement layer close to the folds was thick or multifilamentous (Fig. 4). Several axon terminals whose number was not estimated were present, showing few synaptic vesicles and multivesicular bodies (Fig. 3). Scattered, rare and broad junctional folds containing collagen fibrils were observed associated with these axons (Fig. 5).

## DISCUSSION

Sprouting and degeneration of vertebrate motor nerve terminals have been suggested to occur for a long time [1,4,6,10,26,27,33-37]. Evidence of myoneural junction plasticity has been observed in many physiological situations such as increased activity [30] or inactivity [2,13,29,30], drug blockade [39], environmental changes [22], or pathological conditions [5,7,20]. Irintchev *et al.* [21] observed the reinnervation and recovery of denervated mouse soleus muscle within 5-6 months. The oblique abdominal muscles perform continuous activity related to the fast abdominal movements associated with respiration and support the abdominal viscera during locomotion. In the present study using aging rats, about 50% of the myoneural junctions from rat oblique abdominal muscles presented evidence of reorganization, such as rare nerve terminal sprouting, empty synaptic clefts, enlarged and not packed junctional folds without axon terminal contact, and axons exhibiting few synaptic vesicles, coated vesicles, multivesicular bodies, etc.

Primary synaptic cleft regions devoid of nerve terminals and exhibiting bare junctional folds were clearly demonstrated in this study, and axon remnants in the vicinity as described by Cardasis and Padykula [10] and Cardasis [8] in rat muscles and by Fahim and Robbins [14] in mouse muscles were also observed. In a previous study, we have demonstrated the presence of axons completely or partially engulfed by Schwann cell cytoplasmic projections in the fibularis longus muscle of aged mice [6]. These axons present deep morphological changes such as a lack of synaptic vesicles, swallowed mitochondria, dense bodies and myelin figures, a phenomenon that might be related to axonal degeneration and retraction. It is important to note that the animals used in the present study were between 18 and 24 months old. According to Cardasis and Padykula [10], junctional reorganization occurs at a higher frequency (90% of NMJs) in aging rats than in rats aged 3-5 months (33% of NMJs). The

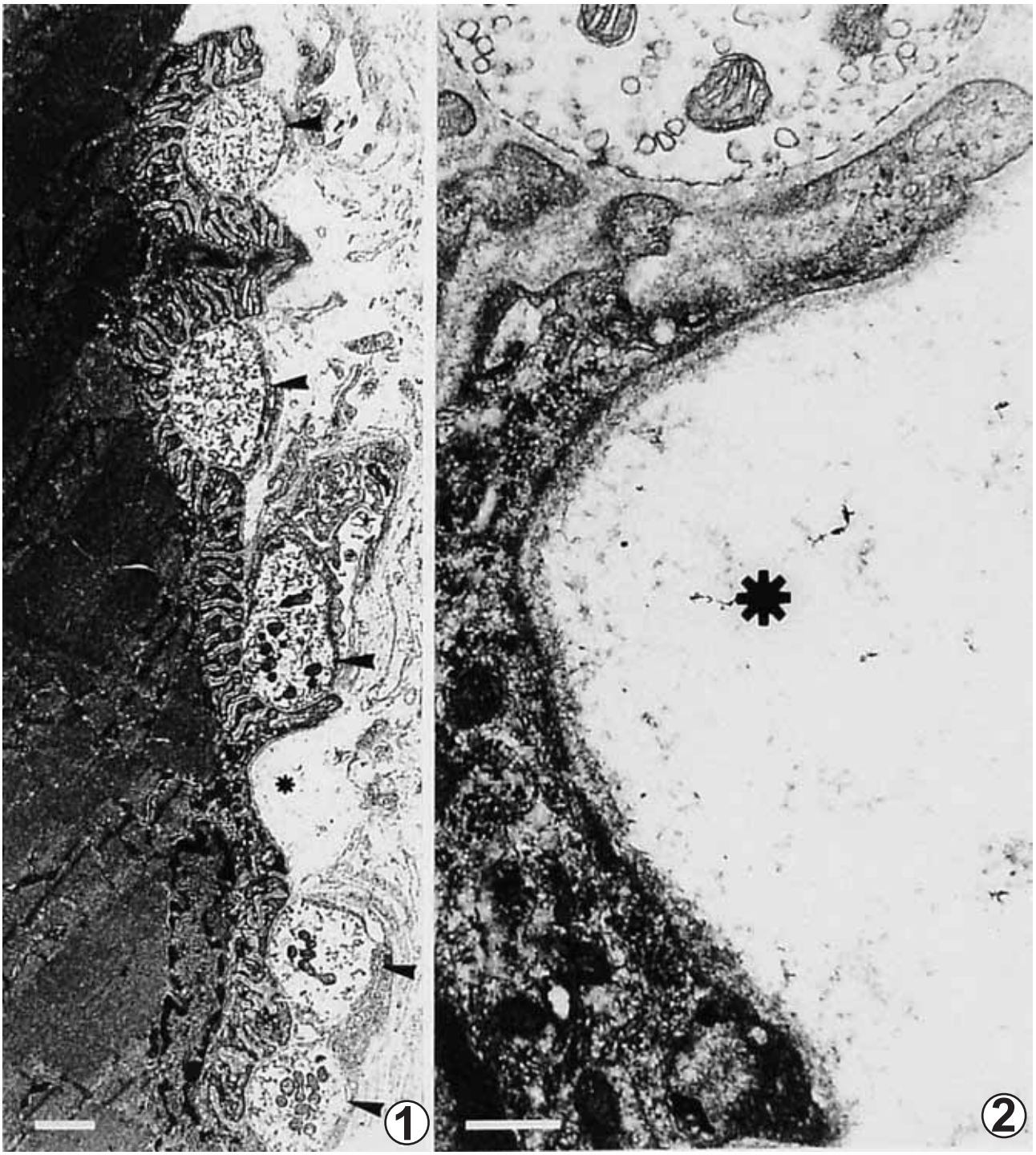


**Figure 1.** Electron photomicrograph of a neuromuscular junction associated with a white muscle fiber. Five axon terminals are seen (**arrowheads**) and one empty space (**asterisk**) where apparently there was another terminal. Two axon terminals (left) show morphological characteristics of normal endings. The terminal axon in the center suggests that it is detaching from the muscle fiber surface and long Schwann cell processes are surrounding it. Bar = 5 μm.
**Figure 2.** Detail of the empty synaptic cleft (**asterisk**) seen in Figure 1. Bar = 1 μm.

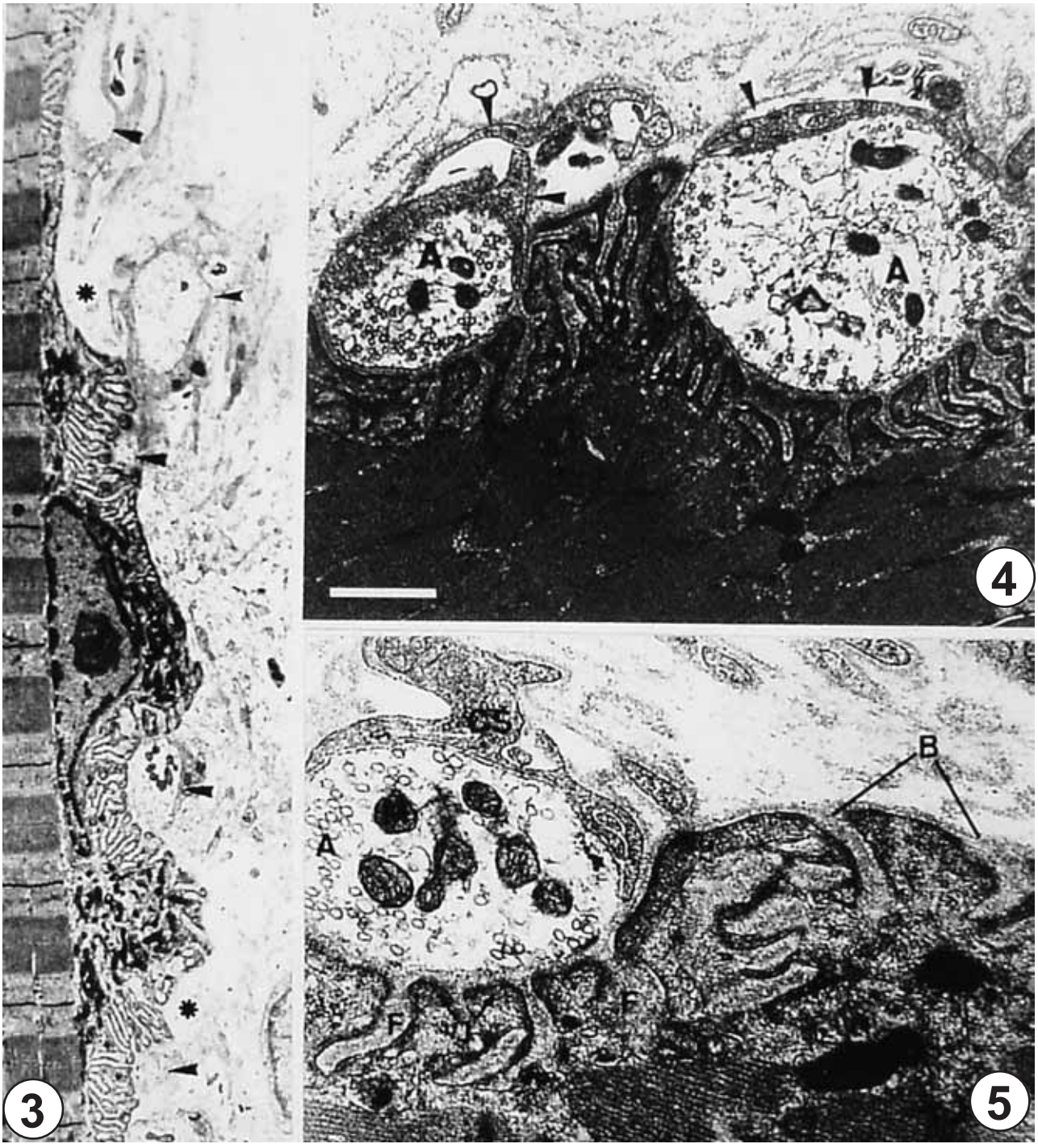


**Figure 3.** Electron photomicrograph of a neuromuscular junction associated with an intermediate muscle fiber. Six axon terminals exhibiting different degrees of abnormalities are observed at this neuromuscular junction (**arrowheads**). The two axon terminals on the top resemble "ghost" images of their primitive structure and the last one on the bottom shows damaged mitochondria and pale cytoplasm. Empty synaptic clefts are marked with asterisks. Bar = 5 μm. **Figure 4.** Electron photomicrograph of a neuromuscular junction with a red fiber. Two axon terminals (**A**) are present and are covered by Schwann cell processes (**arrowheads**). The axon ending on the left is partially separated from the synaptic cleft by a Schwann cell process. Bar = 5 μm. **Figure 5.** Detail of the electron photomicrograph of a neuromuscular junction associated with a red fiber. The axon endings (**A**) present few synaptic vesicles and the Schwann cell process is penetrating the synaptic cleft. On the right, loose junctional folds (**J**) are seen (**B**). Secondary synaptic clefts (**F**) are evident. Bar = 5 μm.

authors emphasize that the relative inactivity of laboratory rats compared to wild rats might change NMJ ultrastructure, particularly in the postural muscles. The same was suggested for adult frogs by Jans *et al.* [22] who demonstrated that the sprouting rate is twice as high in animals kept under natural conditions (season change included) compared to laboratory animals. On the basis of these data, the authors concluded that the remodeling is "inherent" to nerve terminals and sprouting is counterbalanced and reversed by nerve activity. On the other hand, repeated muscle damage in mice achieved by continuous muscle activity leads to changes in soleus muscle fiber composition and consequent enhanced axon sprouting and the formation of new myoneural junctions [38]. Although we present clear evidence of axon terminal retraction in this study, sprouting or branching was not a common finding. These facts are in accordance with the above considerations that age and natural inactivity in laboratory cages lead to an increased axonal retraction which is not counterbalanced by the same intensity of sprouting. Lichtmann *et al.* [26] studied myoneural junctions in sternomastoid muscle of living mice and observed no significant differences during morphological evaluations made at intervals of up to 6 months. Nevertheless, these authors found that the myoneural junctions become enlarged in two dimensions after muscle fiber growth. However, Smith and Rosenheimer [32] suggested that the myoneural junction does not change in size along time in the rat diaphragm. In this respect, Jans *et al.* [22] demonstrated that sprouting and retraction occur during environmental changes and during growth and aging in frog pectoris muscles. Abandoned synaptic clefts and an increase in junction length were observed with age but were independent of growth. Many studies have reported the presence of low enzymatic activity, fragmentation and broken myoneural junctions in old animals [6,11,33]. These morphological signs observed by light microscopy might be associated with a reduction in receptor number or a decreased area of synaptic contact, resulting in a decline in trophic nerve-muscle interaction and impairment of stimulus transmission [3,16,23]. Krause and Wernig [24] reported that after axon retraction the atrophic muscle fiber cannot keep the acetylcholine receptor clusters by itself. According to Cardasis [8], axonal contacts are scarce in old animals and reduce the effective area of synaptic contact in the myoneural junctions.

The presence of numerous coated vesicles in the nerve terminals has been reported by Fahim and Robbins [15] and Cardasis and La Fontaine [9] in old rats. Although we did not quantify them, figures of coated vesicles were common in the terminal axons throughout this study. Most authors agree that coated vesicles may represent a more rapid turnover of synaptic vesicles with increasing age as a compensatory mechanism for the loss of neuromuscular transmission efficiency.

In contrast to our previous observations on *fibularis longus* muscle in aged rats [6] lysosomes and lipofuchsin figures in the terminal axons were not found during this study. Marked abnormalities of the entire myoneural junctions suggesting degeneration, as reported in elderly mice and rats [15,17], were not observed in the present study.

As mentioned earlier [18,19,23], this sprouting and retraction of nerve terminals at the NMJs represent a natural process that occurs throughout the life of the animal. Recent evidence suggests that this process occurs in response to the loss of acetylcholine receptors in the muscle cell membrane [3].

Three conclusions can be drawn from the present results: a) the phenomena associated with junctional plasticity may be restricted to points along the myoneural junction; b) axonal removal is morphologically clear, frequent and well defined in old animals; c) other signs of plasticity such as sprouting were not well characterized in this study and will require additional systematic observations.

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