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Extensive multiple innervation and abnormal synaptogenesis in muscular dysgenesis (mdg/mdg) in the mouse embryo

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Summary. Muscular dysgenesis (mdg) is an autosomal recessive mutation in the mouse characterized by total muscle inactivity *in vivo* or *in vitro*. The muscle fiber in the mdg/mdg diaphragm was not only morphologically abnormal but also multiply innervated ; the motor innervation was very dense, showing overgrowth and sprouting. As expected at the ultrastructural level, nerve-muscle contacts were composed of dense appositions of numerous axon terminals (dense focal polyinnervation). Moreover, these mdg/mdg neuromuscular junctions, lacking post-synaptic unfolding, were immature compared to the control ones. This retarded neuromuscular junction differentiation in muscular dysgenesis may be related to considerable delay in muscle maturation and/or abnormal muscular differentiation, or to a nerve defect independent of, or causally related to, the muscular defect.

Introduction.

Muscular dysgenesis (mdg) is an autosomal recessive mutation in the mouse, characterized by a total lackof contractile activity of the striated (skeletal) musculature (Gluecksohn-Waelsch, 1963 ; Paï, 1965 a, b). The process of muscle differentiation sèems impaired (Paï, 1965 a, b), and ultrastructural studies have shown that the sarcoplasmic reticulum is dilated and the myofibrillar system disorganized (Banker, 1977). As the mutation can be expressed *in vitro* by mdg/mdg muscle cells (Bowden-Essien, 1972) in the absence of nerve cells, the hypothesis of a primary muscle abnormality was proposed and substantiated by electrophysiological studies (Powell and Fambrough, 1973). A recent investigation of mdg/mdg motor innervation demonstrated generalized axonal sprouting and proliferation with multifocal innervation of single muscle fibers (Rieger and Pinçon-Raymond, 1981). As experimentally proven in other systems (Benoit and Changeux, 1975, 1978 ; Brown *et al.*, 1977), these striking innervation

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abnormalities are possibly a reaction of motor innervation to muscle inactivity. However, in this genetic defect in which the molecular target is not known, the motoneurone cannot be excluded as an independent or even, primary, target of the mutation (Rieger *et al.,* 1980). This paper reports ultrastructural studies of mdg/mdg innervation exploring this possibility. Inactivity alone does not prevent normal neuromuscular junction maturation, at least in chick (Freeman *et al.,* 1976) or duck (Sohal, 1981) embryos, and there are cytochemical and histochemical indications of the existence of numerous variably-mature neuromuscular contacts in mdg/mdg embryos before and at birth (Rieger and Pinçon-Raymond, 1981). We thought it important to examine the ultrastructural characteristics of mdg/mdg neuromuscular junctions and compare their state of differentiation with that of their normal counterparts. The aim of our study was twofold : (i) to confirm the dense polyinnervation of mdg/mdg muscle fibers with a high axon terminal density in the same innervation area, (ii) to provide evidence for the existence of very immature neuromuscular contacts on mdg/mdg muscle fibers, suggesting a considerably retarded neuromuscular junction differentiation at embryonic day 18.

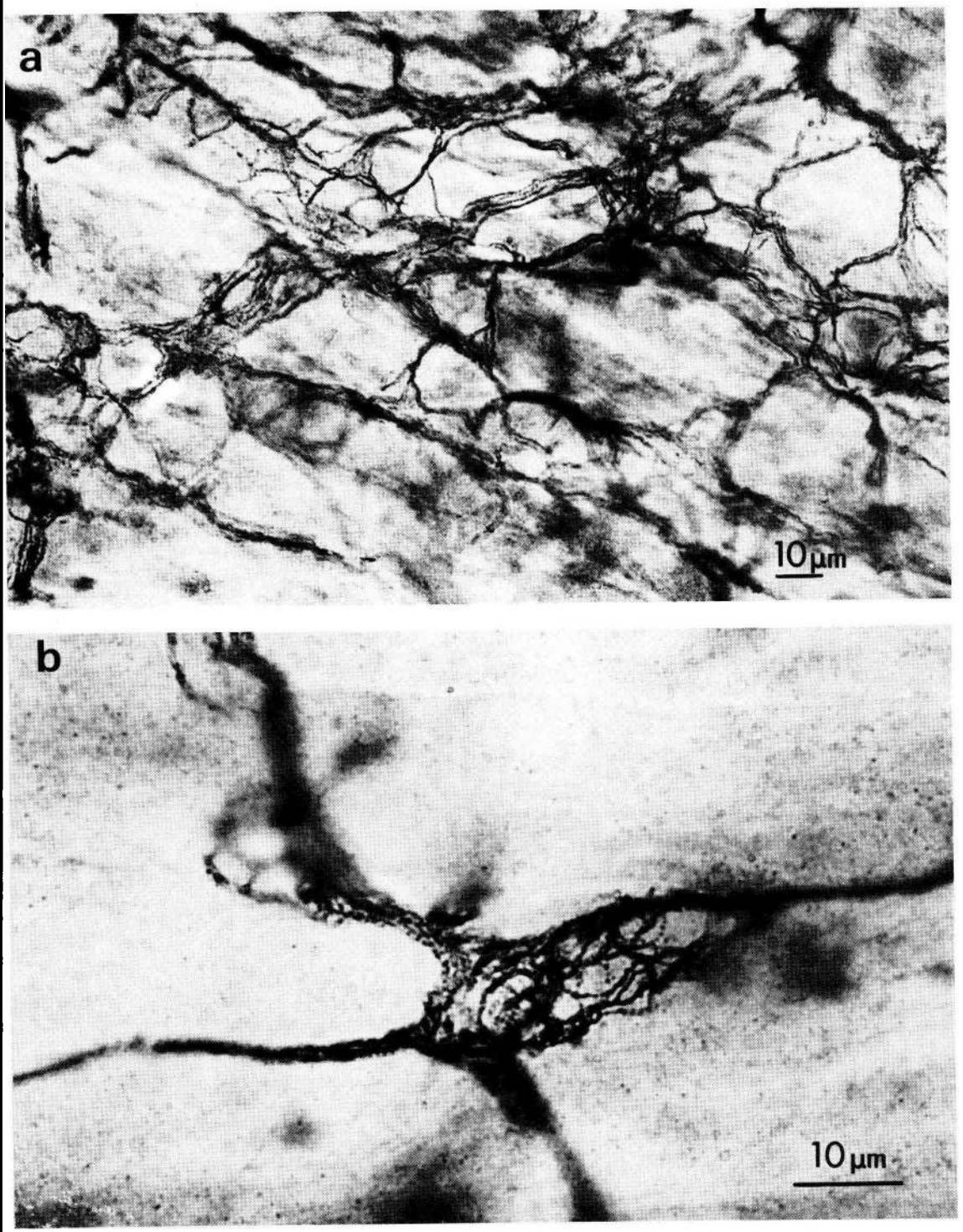
Material and methods.

The muscular dysgenesis (mdg) mutation is maintained on a 129 ReJ genetic background. The original breeding pairs were kindly provided by Dr. J. Powell (Smith College, Northampton, Mass., USA) in 1976 and 1978 and maintained by brother-sister mating and progeny testing in the Children's Hospital of the Harvard Medical School, Boston (Mass.), then later in Paris. Our mdg-bearing mouse colony may be considered as inbred. Dated pregnancies were obtained after one night of mating, and the day the vaginal plug was seen was chosen as day 0 of gestation (average gestation time : 19.5 days). The pregnant females were sacrificed by cervical dislocation and the embryos or fetuses collected by cesarean delivery. Whole diaphragms were dissected out and kept extended to their original dimensions ; the superficial membranes were stripped away with fine insect pins.

The tissues were processed as reported by Rieger and Pinçon-Raymond (1981) for neurofibrillary staining, according to a combination of the methods of Barker and Ip (1963, 1965) and Gladden (1970). They were fixed for electron microscopy (Fardeau *et al.,* 1978) by immersion in 2.5 p. 100 glutaraldyhyde for

FIG. 1. — *Some aspects of nerve overgrowth, sprouting and multiple innervation in mdg/mdg diaphragm.* Embryonic day 18 (E 18).

a : Collateral and ultraterminal sprouting (low magnification) ;
b : An area of dense focal polyinnervation (high magnification).


a
10μm
b
10μm

1 h at room temperature, then in 0.6 p. 100 glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, overnight at 4-6 °C. Ultrathin sections were made (LKB ultratome) after semithin sections 1-μm thick had been cut, then stained in toluidine blue (borax buffer) to select the longitudinal orientation of the muscle fibers and the neuromuscular contacts. Staining was performed with a saturated solution of uranyl acetate in acetone, followed by staining with a solution of lead citrate. Observations were made with a Philips EM 300 electron microscope (accelerating voltage 60 kV, objective aperture : 20 μm).

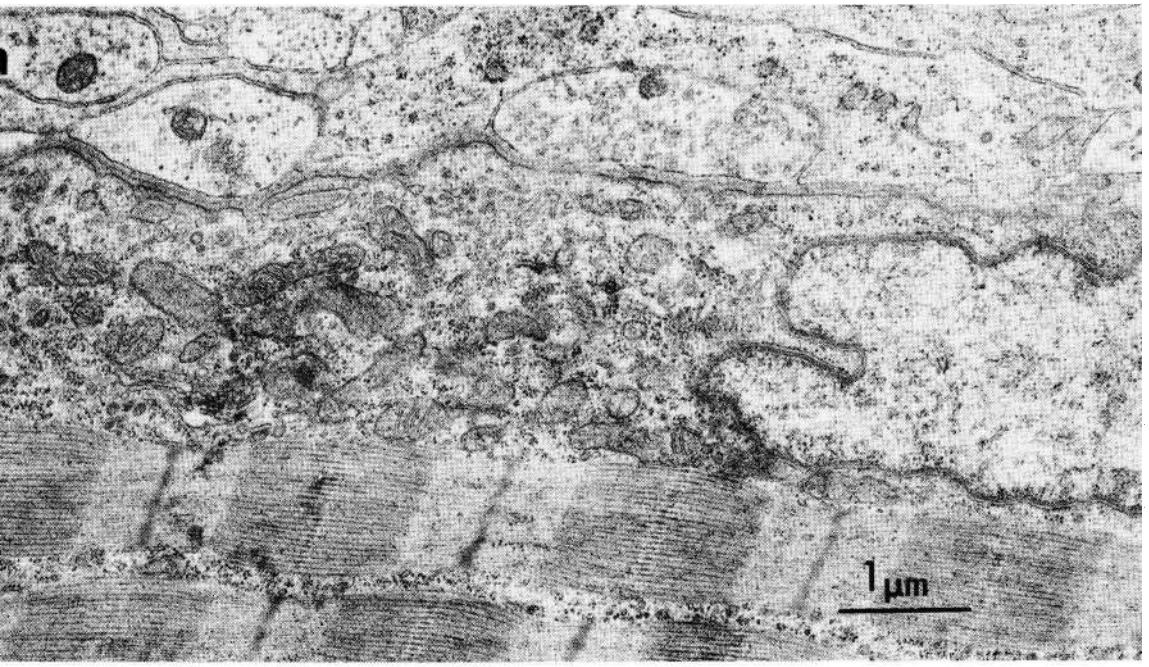
Results.

1) *High density of the axonal network of phrenic (motor) innervation in mdg/mdg diaphragms (silver nitrate staining).* — As already demonstrated at birth, the motor innervation of mdg/mdg diaphragms at embryonic day 18 (E18) shows a very dense nerve overgrowth (fig. 1 a) with generalized collateral and ultraterminal sprouting. Numerous innervation sites on muscle fibers were highly complex with thin intertwinned terminal branches corresponding to focal poly-innervations of a high order (fig. 1 b).

2) *Ultrastructural correlates of mdg/mdg nerve outgrowth and multiple innervation ; mdg/mdg neuromuscular junctions.* — The present study was performed on two different mdg/mdg diaphragms and two (+ mdg ?) control diaphragms. We analyzed 17 different innervation areas in the mdg/mdg diaphragms and 8 neuromuscular junctions in +/mdg diaphragms. The E18 control neuromuscular junctions were very homogeneous, showing rather well-differentiated morphological maturation (fig. 2 a). Their distinctive feature were : continuous post-synaptic thickening, well-established primary synaptic folds and secondary synaptic folds in the process of formation. Several axon terminals, observed at the same innervation site, possibly reflected some state of poly-innervation. The mdg/mdg innervation sites differed greatly in two main aspects (fig. 3 a, b), *i.e.* we found numerous axon terminals filled by synaptic vesicles but we did not observe secondary synaptic folds. These morphological aspects indicated that the innervation density of the mdg/mdg neuromuscular junctions was higher and their morphological maturation less advanced than normal +/mdg ? neuromuscular junctions. Occasionally, the basal lamina of the muscle fiber appeared to be less defined. The pre-synaptic membrane of the mdg/mdg axon terminals sometimes presented a much less defined or organized structure than observed for the normal pre-synaptic membrane (fig. 4).

FIG. 2. — *Ultrastructure of control E 18 +/mdg ? neuromuscular junctions.*

a : General view (low magnification) ;
b : Developing secondary post-synaptic unfolding (★ : high magnification).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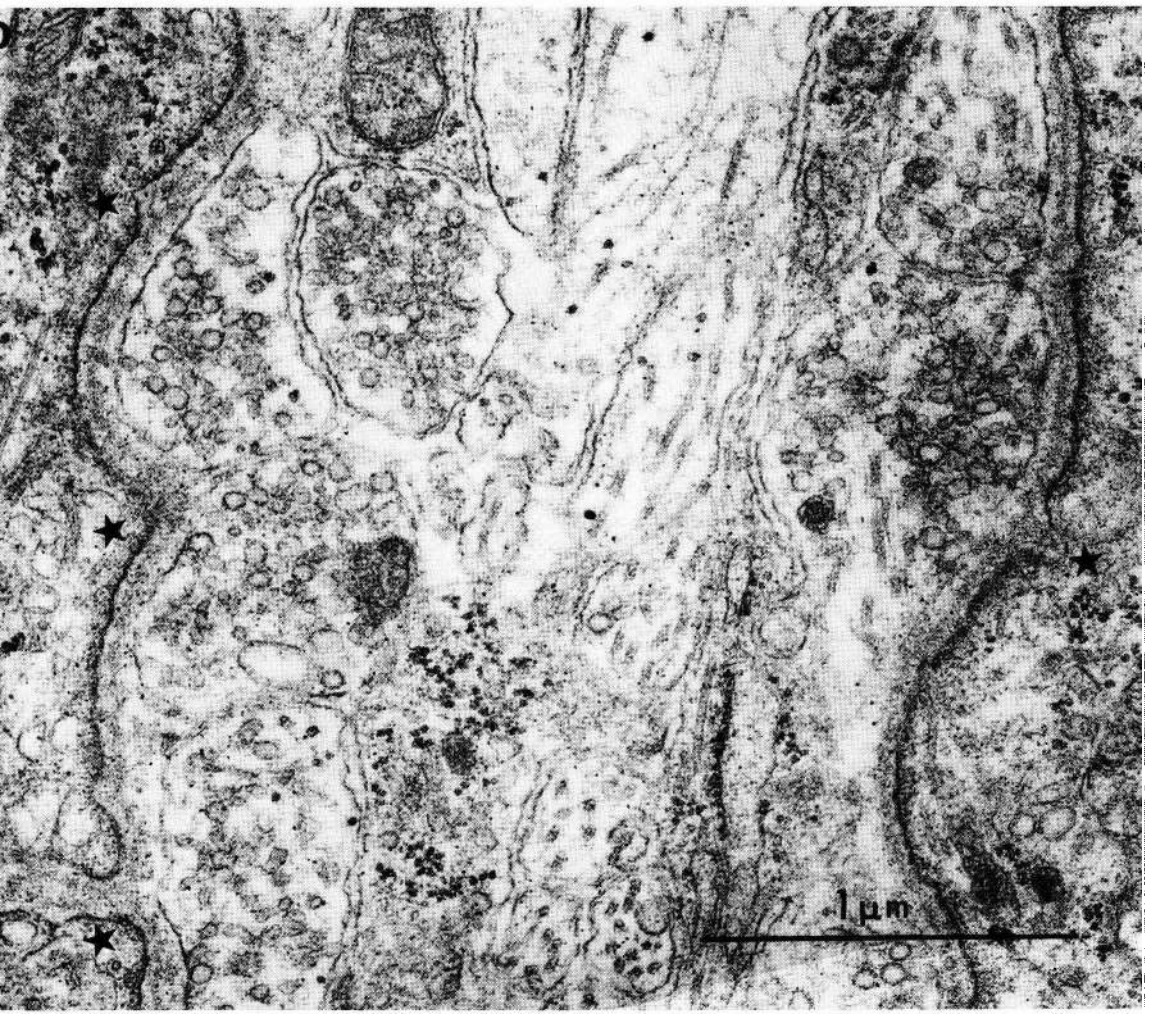
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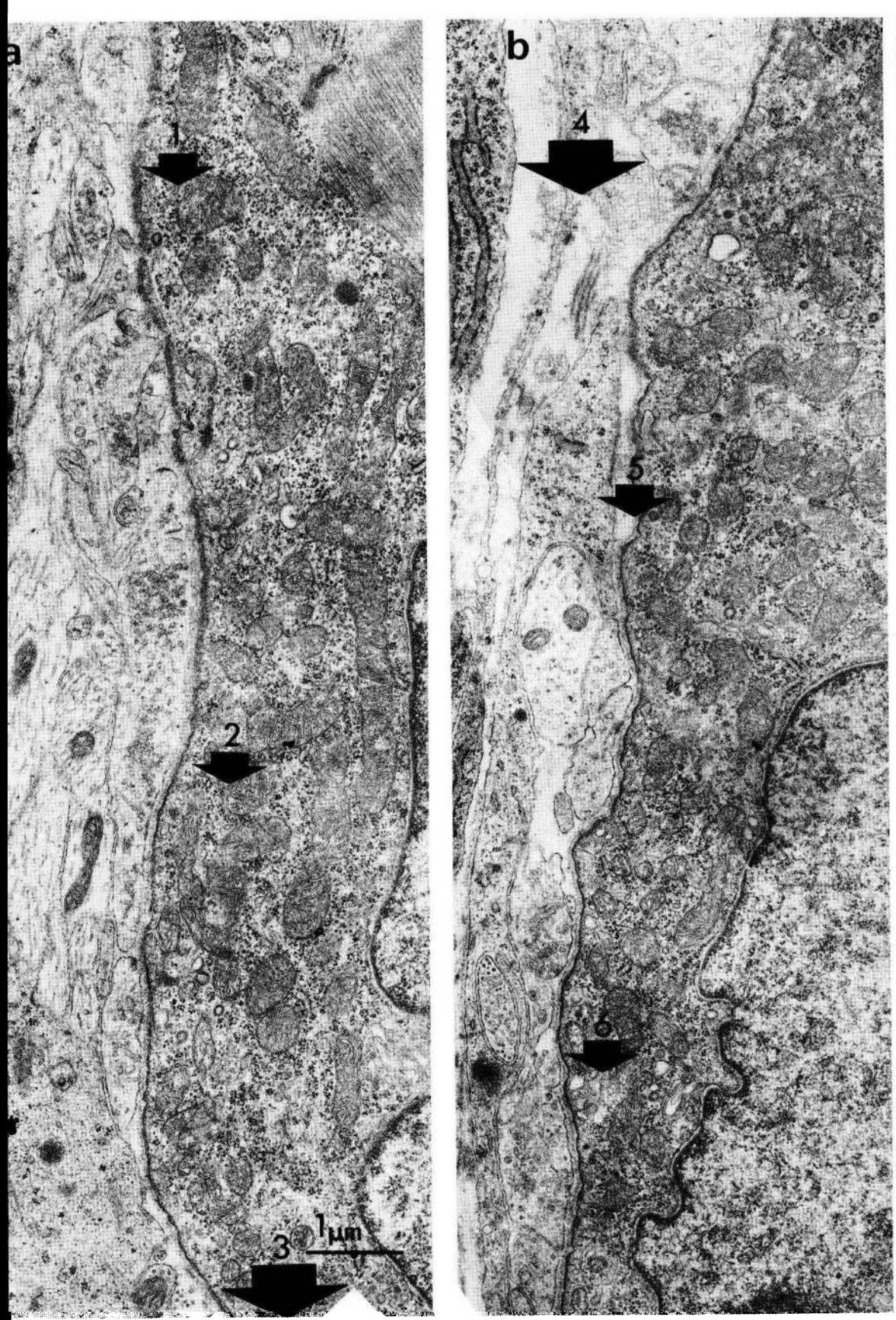
Discussion.

Muscular dysgenesis has long been thought to be primarily a pure embryonic muscle hereditary defect, essentially because the mutation is expressed *in vitro* in primary muscle cell cultures (Bowden-Essien, 1972) with a total lack of muscle contractile activity. Mainly using morphological or ultrastructural criteria, previous authors have stressed the abnormality or « deviation » (Banker, 1977) of muscle development. Muscle inactivity has been shown to determine nerve reactions, and ultraterminal sprouting has been observed in experimental or hereditary conditions, such as after botulinum or tetanus toxin poisoning or in motor end-plate disease (Duchen and Strich, 1968 ; Duchen, 1970 ; Duchen and Tonge, 1973 ; Rieger and Pinçon-Raymond, 1980 ; Pinçon-Raymond and Rieger, 1981). We have reported evidence of such nerve ultraterminal sprouting in muscular dysgenesis, characterized by a dense, plexiform nerve overgrowth and collateral sprouting and multifocal innervation of muscle fibers (Rieger and Pinçon-Raymond, 1981). Given such marked abnormalities, we wished to explore the possibility of an independent expression of the mutation directly in the nerve or the motoneurone. In view of the results reported in chicken and duck embryos (Freeman *et al.*, 1976 ; Sohal, 1981), in which pharmacologically-induced muscle inactivity determined the maintenance of polyneuronal innervation but did not impair the formation and maturation of neuromuscular junctions, we evaluated the morphological characteristics of motor innervation and neuromuscular junctions at the ultrastructural level. We found the expected ultrastructural aspects, *i.e.* dense polyinnervation of the innervation sites in E18 mdg/mdg diaphragms. Also, in rather mature-looking mdg/mdg muscle fibers with peripheral nuclei and organized myofibrils, we found clear evidence of delayed maturation of the post-synaptic membrane which totally lacks secondary synaptic folds. Our observations are in agreement with the results reported by Banker (1977) on limb muscles. This author interpreted the retarded development of the mdg/mdg motor end-plates as secondary to poor muscle development. However, as an alternative interpretation of retarded development, we suggest to extend the conclusions of Freeman *et al.* (1976) and Sohal (1981) to the mammalian neuromuscular system : muscle inactivity alone does not provoke or cannot explain the immaturity of the mdg/mdg neuromuscular contacts. Moreover, we

FIG. 3. — *An E 18 mdg/mdg neuromuscular junction : extended innervation area with dense polyinnervation.*

Note the extremely wide myofiber area occupied by many nerve terminals. No post-synaptic unfolding. The innervation territory is divided in two ; the arrows and numbers on the micrographs indicate the points in question.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observed structural abnormalities of the mdg/mdg pre-synaptic membrane and muscle basal lamina not reported in situations of muscle inactivity. No general membrane alteration has been detected so far by electrophysiological studies (Powell and Fambrough, 1973 ; Bournaud, 1980 ; Rieger *et al.*, 1980). Other evidence for considering the mdg/mdg nerve as a possible independent, if not primary, target of the mutation comes from *in vitro* studies demonstrating that the mdg phenotype (absence of contractile activity) may be reversed by co-culturing the mdg/mdg muscle cells with dissociated cells from normal spinal cord (Koenig *et al.*, 1980).

Considering all these results, it is probable that muscle inactivity is responsible for maintaining a high degree of multiple innervation in the mdg/mdg muscle fiber but that some nerve defect, either dependent or independent of the muscular expression of the mutation, is revealed in the marked ultrastructural abnormalities of the mdg/mdg neuromuscular junction. One direct approach to a precise evaluation of such a possibility would be to provoke muscle inactivity in the normal mouse embryo by injecting AChR blockers, such as α-bungarotoxin or curare, at the earliest time of muscle activity, *i.e.* embryonic days 12 to 14.

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Résumé. La mutation autosomale récessive « muscular dysgenesis » (mdg), chez la souris, se caractérise par l'absence de toute activité contractile musculaire squelettique, chez l'embryon, qui meurt à la naissance. Le défaut génétique s'exprime comme un retard important de la différenciation et de la maturation des fibres musculaires et de la synaptogénèse, avec une multi-innervation très dense. La jonction nerf-muscle présente des aspects ultrastructuraux d'immaturité très prononcée (absence de plis post-synaptiques secondaires, anomalies de la lame basale musculaire, etc.) qui ne s'expliquent pas simplement comme une conséquence directe d'une inactivité primaire. Un ensemble d'études cytologiques, ultrastructurales et biochimiques permettent de proposer que la mutation « muscular dysgenesis » affecte le développement embryonnaire de *l'ensemble* du système neuromusculaire, sans préjuger de ce qu'est la cible primaire, muscle *ou* nerf (motoneurone).

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FIG. 4. — *Details of the mdg/mdg neuromuscular junction.*

There is no trace of any post-synaptic unfolding. The basal lamina of the muscle fiber appears to be less dense than in the control neuromuscular junction (fig. 2 b). A Schwann cell process in apposed to the myofiber (*).

1 µm

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