Spinal cord-muscle relations: their role in neuromuscular development in birds

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ABSTRACT In the present study we focused our attention on the role of spinal cord-muscle interactions in the development of muscle and spinal cord cells. Four experimental approaches were used: 1) muscle fiber-spinal cord co-culture; 2) chronic spinal cord stimulation in chick embryos; 3) direct electrical stimulation of the denervated chick muscle; 4) skeletal muscle transplantation in close apposition to the spinal cord in chick embryos. The characteristics of mATPase and energetic metabolism enzyme activities and of myosin isoform expression were used as markers for fiber types in two peculiar muscles, the fast-twitch PLD and the slow-tonic ALD. *In vitro,* in the absence of neurons, myoblasts can express some characteristics of either slow or fast muscle types according to their origin, while in the presence of neurons, muscle fiber differentiation seems to be related to the spontaneous rhythm delivered by the neurons. The *in ovo* experiments of chronic spinal cord stimulation demonstrate that the differentiation of the fast and slow muscle features appears to be rhythm dependent. In the chick, direct stimulation of denervated muscles shows that the rhythm of the muscle activity is also involved in the control of muscle properties. In chick embryos developing ALD, the changes induced by modifications of muscle tension demonstrate that this factor also influences muscle development. Other experiments show that muscle back-transplantation can alter the early spinal cord development.

KEY WORDS: *spinal cord, skeletal muscle, muscle fiber differentiation, avian embryo, chick*

Introduction

In vertebrates, tissular interactions have long been recognized to be critical in ontogeny. Embryonic development relies both on intrinsically programmed sequences of differentiation in individual cells and on interactions within or between tissues. Changes in the relations between the embryonic cells can modify their differentiation, and certain developing systems are particularly valuable for studying this phenomenon. This is the case with the spinal cord-skeletal muscle interactions. Much of the research designed to clarify this problem has been carried out on birds (in embryonic and postnatal development). Our experiments were undertaken to elucidate the mechanisms involved in the phenotypic diversification in muscle fiber types. Such studies were facilitated by the presence in chicken of muscles predominantly containing one type of fiber – the *anterior latissimus dorsi*, or ALD, a muscle primarily composed of two types of slow-tonic fibers termed III A and III B, and the *posterior latissimus dorsi* or PLD almost exclusively composed of two fast-twitch fiber types termed II A and II B (Ashmore and Doerr, 1971; Asiedu and Shafiq, 1972; Ashmore *et al.*, 1978). In the ALD muscle fibers two slow native isomyosins designated SM1 and SM2 are expressed, SM2 predominating in the adult. PLD muscle fibers contain three fast native isomyosins FM1, FM2, FM3 (d'Albis *et al.*, 1979; Hoh, 1979). In addition these two fiber types – slow-tonic and fast-twitch – differ in their innervation pattern, the fast-twitch fibers being focally innervated by one or only a few nerve terminals and the slow-tonic fibers innervated by several nerve terminals at distributed terminals (Ginsborg and Mac Kay, 1961; Hess, 1961).

Abbreviations used in this paper:AChE: acetylcholinesterase; ALD, *Anterior Latissimus Dorsi*; CPK, creatine phosphokinase; FM1, FM2, FM3, fast native isomyosins; LC1F, LC3F, fast myosin light chains; LC1S, slow myosin light chain; mATPase, myofibrillar adenosine triphosphatase; MDH, malate dehydrogenase; MLC, myosin light chains; NADH-TR, nicotinamide adenine dinucleotide tetrazolium reductase; PLD, *Posterior Latissimus Dorsi*; SM1, SM2, slow native isomyosins.

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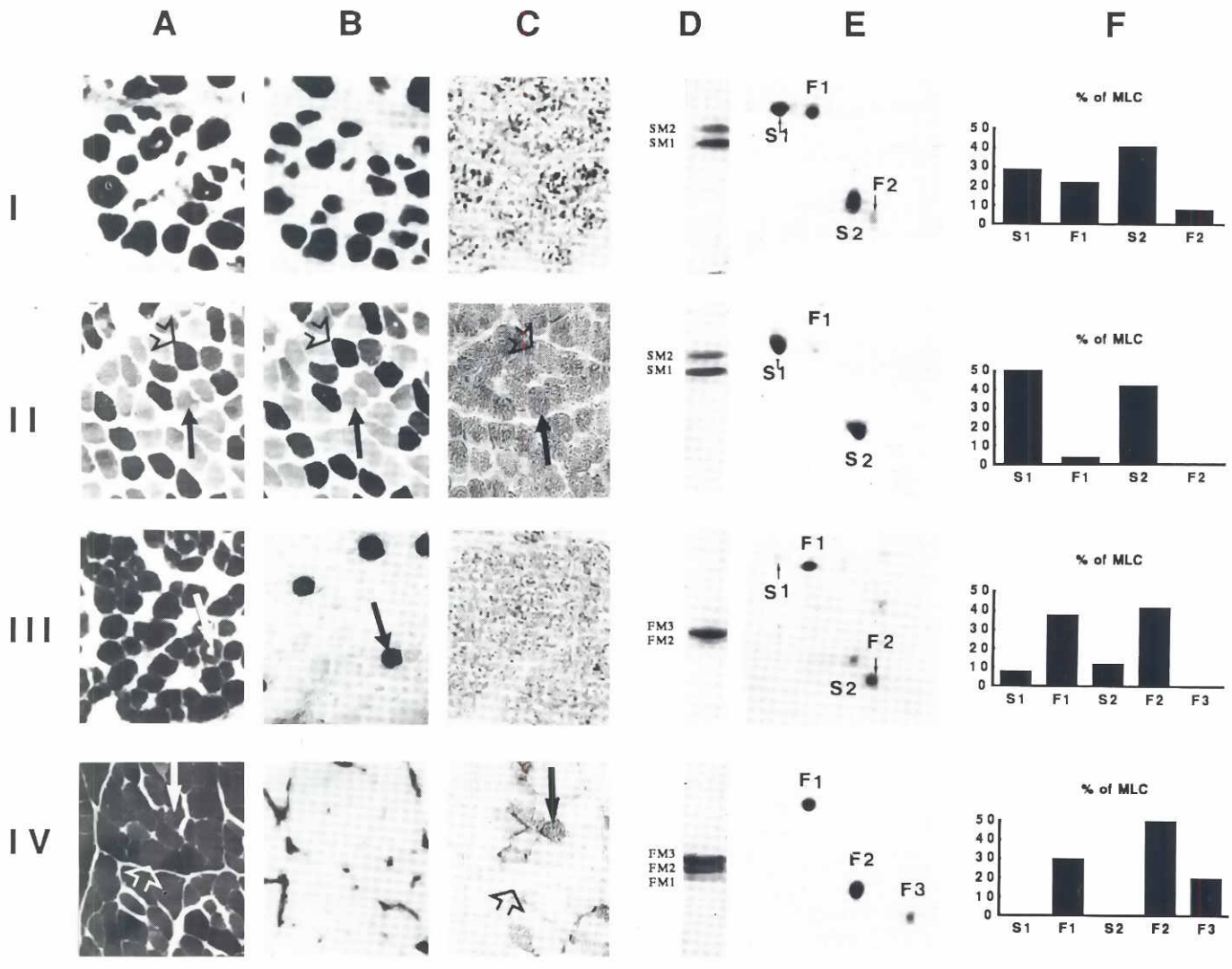

Fig. 1. ***Latissimus dorsii* muscle characteristics.** *Each horizontal line* **(I, II, III, IV)** *represents: muscle serial transverse sections stained for mATPase following (A) alkali or (B) acid preincubations and for (C) SDH activity; (D) non-denaturing gel electrophoresis of myosin; (E) two-dimensional gel electrophoresis of myosin light chains (MLC); (F) relative proportion of each MLC. In line* **(I)** *ALD muscle from 14-day-old embryo,* **(II)** *ALD muscle from 2-week-old chicken,* **(III)** *PLD muscle from 14-day-old embryo,* **(IV)** *PLD muscle from 2-week-old chicken. In* **(I)** *all fibers are of type IIIB (x380). In* **(II)** *arrows indicate type IIIA and arrowheads type IIIB fibers (x190). In* **(III)** *nearly all fibers are of type II, arrows indicate some type I fibers (x380). In* **(IV)** *arrows indicate type IIA and arrowheads type IIB fibers (x190). SM1 and SM2= slow native isomyosins. S1 and S2= slow MLC; F1, F2 and F3= fast MLC.*

During chick embryonic development the different fiber types have been essentially characterized by their myofibrillar ATPase (mATPase) and their myosin composition. Numerous studies (Khaskiye *et al.*, 1980; Butler *et al.*, 1982; Laing and Lamb, 1983; Phillips and Bennett, 1984; Crow and Stockdale, 1986; Sohal and Sickles, 1986) have shown that at early stages of development the fibers appear intrinsically determined whereas later on their development requires the presence of functional innervation (Harris, 1981; Mc Lennan, 1983; Gauthier *et al.*, 1984; Crow and Stockdale, 1986). In order to know by what mechanisms the motor neuron finally controls muscle differentiation, a chronic electrical stimulation of the brachial zone of the spinal cord – the zone innervating the ALD and PLD muscles – was first carried out in chick embryos. The second experimental approach consisted in studying the role of the nerve in the differentiation of muscle fibers in an *in vitro* system, and myoblast development was examined respectively in the absence and presence of motor neurons. To see whether the muscle activity influences the postnatal development of the slow and fast chick muscles, direct stimulation of the denervated muscles was performed. These procedures allowed us to demonstrate the important role played by the presence of functional motor neurons on muscle differentiation. In order to analyze the possible effects of embryonic muscle tissues on spinal cord development *in ovo*, another experimental approach was used. Embryonic skeletal muscles were placed in close apposition to the neural tube in chick embryos. Our observations demonstrate that the action of these grafts first results in an increase in the mitotic activity of the neuroepithelial cells and in the modification of the number of motor neurons.

Results

Differentiation of slow and fast muscle characteristics during chick development

Presumptive slow and fast muscle fibers of the slow-tonic ALD and fast-twitch PLD muscles can be respectively distinguished in embryonic development on the basis of their myofibrillar ATPase activity (mATPase) and myosin composition (Fig. 1).

Slow-tonic ALD muscle

As early as day 8 of incubation all ALD muscle fibers appear darkly stained after mATPase with either alkaline or acid preincubation. They can be considered as the slow-tonic IIIB or ß' fibers according to the nomenclatures respectively established for the adult muscle fibers by Barnard *et al.* (1982) and Ashmore *et al.* (1978). The second type of slow-tonic fibers, called IIIA or α', characterized by a low level of mATPase after either alkaline or acid preincubation, becomes detectable at the end of incubation. Whereas by day 14, fast II or α fiber type (of which mATPase activity is acid-labile and alkali-resistant) can be seen in this slow muscle and its proportion never exceeds 5% at any developmental stage (Toutant *et al.*, 1979, 1980b). This enzymatic profile may be related to the composition in native isomyosins since in addition to the two slow native isomyosins SM1 and SM2 that represent the major components of the ALD myosin, trace amounts of fast isomyosins can be detected. But in terms of myosin light chain (MLC) composition, the embryonic ALD contains large amounts of fast isoforms (40 % of the total MLC at the 12th day). This suggests the existence of myosin hybrids composed of slow myosin heavy chains associated with both slow and fast MLC at early stages, the fast components being progressively replaced by slow MLC during embryogenesis (Gardahaut *et al.*, 1985, 1988b).

At the end of the embryonic period, the differentiation of the slow ALD muscle is not completely achieved, as shown by the increase in the oxydative activities in IIIA fibers (Khaskiye *et al.*,1987b), the disappearance of the fast MLC, of the slow isomyosin SM1 and the predominence of the slow native isomyosin SM2, all of which occur during the post-hatching period (Gardahaut *et al.*, 1985, 1988b).

Fast-twitch PLD muscle

By day 8 *in ovo*, the mATPase activity of most PLD muscle fibers is intense after alkaline preincubation and inhibited by acid preincubation, a proof that these fibers belong to the II or α type (Toutant *et al.*, 1979). From the 10th day of incubation onward, some slow fibers exhibiting both alkali-stable and acid-stable mATPase differentiate, and consequently they can be designated as I or ß type fibers. Their maximum number is attained by day 14-15 of embryonic development and decreases later on (Renaud *et al.*, 1983).

These results are in agreement with those obtained concerning the distribution of the different myosin isoforms. Thus in PLD muscle that predominantly contains the fast native isomyosins FM3 and FM2, small amounts of slow isomyosins are also present between day 11 and day 16 of embryogenesis. During this period both slow and fast MLC are expressed, while the fast MLC, LC3F, cannot be detected before day18. Later on, the accumulation of the slow components markedly decreases (Gardahaut *et al.*, 1985, 1988b).

During the first two weeks of post-hatching development of PLD muscle, two subtypes of fast fibers occur: the IIB type, which is characterized by a low level of oxidative activity, and the IIA type, by a high level of oxidative activity. From day 14 onward the PLD muscle is composed of a large majority of IIB fibers as in the adult (Khaskiye *et al.*, 1987b). In addition, a change in the electrophoretic pattern of native isomyosins and of MLC occurs: the relative proportion of FM1 and FM2 isoforms and of the fast MLC LC3F increases while the relative proportion of FM3 decreases. The adult pattern of the fast myosin molecule is established at the end of the first month after hatching (Gardahaut *et al.*, 1988b).

Influence of motor neuron activity upon slow and fast muscle differentiation in the chick embryo

Since in the adult, numerous studies on denervated, cross-innervated and stimulated muscles have demonstrated that muscle properties are regulated by nerve, it was of interest to determine the role of motor neurons in the appearance of the slow and fast characteristics of muscle fibers during ontogenesis. For this purpose, the effects on histochemical features and MLC expression of chronic electrical stimulation of the brachial spinal cord, responsible for the ALD and PLD muscle innervation, were studied in the chick embryo (Fig. 2A, B, C).

Slow-tonic ALD muscle

While a chronic stimulation at low frequency does not seem to affect the enzyme activities and the pattern of MLC (Gardahaut *et al.*, 1985; Fournier Le Ray *et al.*, 1986), stimulation at a fast rhythm performed from day 7 up to day 18 modifies the embryonic development of the ALD muscle. In these conditions, at day 18, the ALD muscle exhibits a large proportion of fast fibers (II type), an increase in fast MLC content and a large decrease in half time to peak of tetanic contraction. When stimulation starts at day 8, no fast type fibers are observed and the fast MLC accumulation is much lower than in the previous series. When chronic stimulation at a fast rhythm is initiated at day 10, the ALD muscle differentiation is not at all modified. These results show that a fast pattern of motor neuron activity induces some slow-to-fast transformations in ALD muscle fibers that occur only when the rhythm modification is initiated before day 10 (Fig. 2A) (Fournier Le Ray *et al.*, 1989).

Fast-twitch PLD muscle

The chronic spinal cord stimulation of the fast PLD muscle at a low rhythm delivered from day 10 to day 19 alters its histochemical profile. In these experimental conditions the slow fiber population (I type) that regresses in the normal course of ontogeny is stabilized (Renaud *et al.*, 1983). The total amount of slow MLC is significantly higher. For instance, in 16-day-stimulated embryo the proportion of slow isoforms reaches 30% of the MLC while the proportion of these isoforms is only about 2% in the control muscle (Fig. 2B) (Gardahaut *et al.*, 1985). These results indicate that spinal cord stimulation at low rhythm induces changes in fast properties turning them into the slow ones in the fast PLD muscle. The decrease in activity of the lactate dehydrogenase (LDH), an enzyme of anaerobic glycolysis and of creatine phosphokinase (CPK) resulting from this stimulation is consistent with this conclusion (Fournier Le Ray *et al.*,1986).

Furthermore the role of motor neuron activity upon the pattern of endplate formation during embryonic development was investigated. The application of spinal cord stimulation at a low frequency results in the appearance of several sites of acetylcholinesterase activity, of multiple acetylcholine receptor clusters and in the

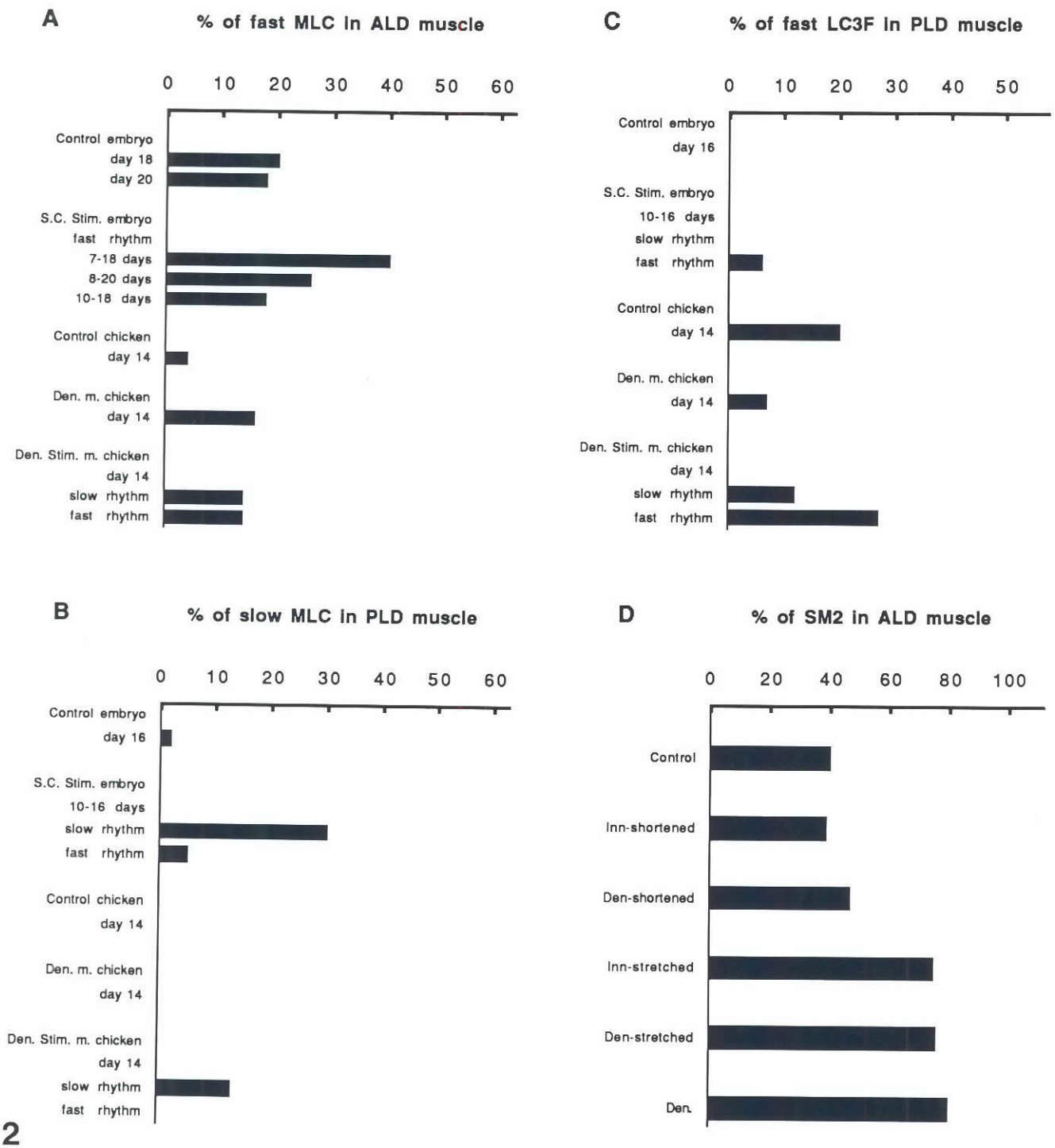

Fig. 2. Influence of activity and tension on myosin isoform accumulation in *Latissimus Dorsii* muscle. (A-C) *Influence of the rhythm of neural and muscle activities on MLC accumulation during embryonic and postnatal development. S.C. Stim. embryo= spinal cord-stimulated embryo; Den. m. chicken= denervated chicken muscle; Den. Stim. m. chicken= denervated-stimulated chicken muscle.* **(D)** *Effects of nerve and tension on SM2 isomyosin accumulation in the slow ALD muscle of 14-day-old chicks. Den.= denervated; Inn.= innervated.*

presence of numerous nerve terminals in the normally focally-innervated PLD muscle fibers (Renaud *et al.*, 1978; Toutant *et al.*, 1980a, 1981b). These results demonstrate that in the chick embryo a change in the activity of motor neurons modifies the development of the innervation pattern in PLD muscle fibers. Moreover, the spinal cord stimulation accelerates the growth of

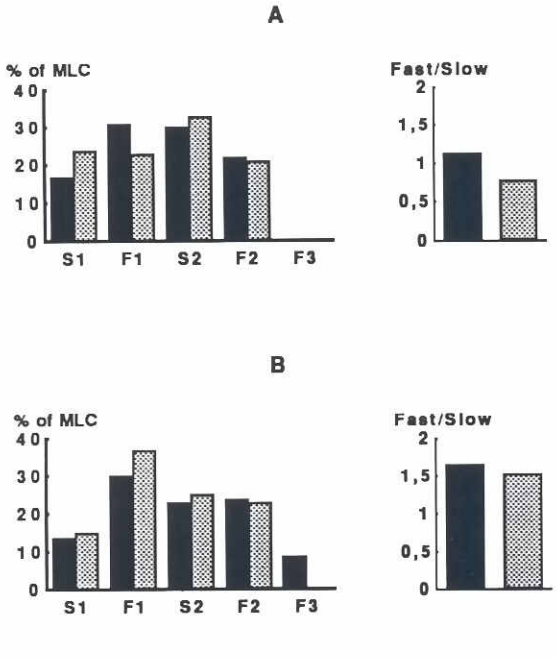

Fig. 3. Accumulation of MLC isoforms in muscle fibers differentiated *in vitro* for 5 weeks. (A) *ALD-derived muscle fibers,* **(B)** *PLD-derived muscle fibers. On the left the histograms show the relative proportion of each MLC isoform. On the right the histograms show the ratio of fast/slow MLC. Black bars represent aneural cultures, dotted bars represent co-cultures. S1 and S2= slow MLC; F1, F2 and F3= fast MLC.*

axons, enhances Schwann cell mitosis and the enclosure of a single axon per Schwann cell, i.e., the process of myelination (Toutant *et al.*, 1981a). Consistent with this idea is our finding that the choline acetyltransferase activity in PLD muscle nerve endings is higher when spinal cord stimulation at a low rhythm is delivered during embryogenesis (Gardahaut *et al.*, 1983).

It was also shown that spinal cord stimulation at fast rhythm accelerates the accumulation of the fast MLC, LC3F, within the fast PLD muscle. This MLC, which is not detected before day 18 during normal embryogenesis, accumulates as early as day 16 in the PLD of spinal cord-stimulated embryos (Fig. 2C) (Gardahaut *et al.*, 1988a). These observations suggest that a fast rhythm of activity enhances PLD muscle differentiation towards the fast adult phenotype.

Influence of muscle activity on the post-hatching maturation of slow and fast chick muscles

Another step of our work consisted in studying the role of muscular activity in the postnatal development of the ALD and PLD chicken muscles. Direct electrical stimulation of the denervated muscles was performed from the second day after denervation, i.e. at day 4 of post-hatching development (Khaskiye *et al.*, 1986). Contractile properties, enzyme activities, MLC expression and molecular forms of acetylcholinesterase (AChE) were analyzed in the slow and fast muscles of experimental chickens.

Slow-tonic ALD muscle

In slow ALD muscle, denervation results in incomplete relaxation, a decrease in the activity of malate dehydrogenase (MDH), an enzyme of the citric acid cycle, and of CPK, an increase in fast MLC accumulation (Fig. 2A) and in the disappearance of the asymetric AChE forms. Direct stimulation of the denervated muscle at either fast or slow rhythm prevents the effects of denervation on relaxation and on CPK activity but has no effect on MDH activity, on fast MLC accumulation (Fig. 2A) and on AChE form expression. Moreover, the direct stimulation of the denervated ALD muscle causes a rhythm-dependent change in tetanic contraction (Khaskiye *et al.*, 1987a; Khaskiye and Renaud, 1988).

Fast-twitch PLD muscle

The main changes in fast-twitch PLD muscle properties following denervation are a slowing down of the time course of the twitch, incomplete relaxation, a decrease in the activity of LDH, of CPK, and in fast LC3F accumulation (Fig. 2C). Denervation induces changes in AChE molecular form distribution, the loss of asymetric forms and the increase in globular forms.

The stimulation of the denervated muscle at a high frequency partly prevents the effects of denervation, results in a time course of the twitch similar to that of the normal muscle, in a large accumulation of LC3F (Fig. 2C), and avoids the disappearance of asymetric forms and the increase in globular forms of AChE. A low frequency stimulation does not restore the twitch time to peak, but increases MDH activity and induces synthesis of slow MLC (Fig. 2B), gives rise to an AChE molecular form pattern characterized by a high proportion of globular forms (Khaskiye *et al.*, 1987a; Khaskiye and Renaud, 1988).

Taken together these results show that denervated ALD muscle does not respond to electrical stimulation as well as PLD muscle does. The different response of these two muscles may reflect the existence of intrinsic properties of the fast PLD and the slow ALD muscles.

Influence of nerve and tension on posthatching maturation of slow chick ALD muscle

The effects of increased or reduced tension on muscle weight, histochemical characteristics, and isomyosin composition were examined in both innervated and denervated muscles (Fig. 2D). It was shown that in both innervated and denervated ALD muscle, a shortening causes atrophy and inhibition of IIIA fiber differentiation. In denervated as well as in innervated ALD, a stretch induces hypertrophy, transformation of all III B fibers, an increase in SM2 isomyosin expression and a decrease in Ca^{2+}-activated myosin ATPase. These results indicate that stretch accelerates the postnatal differentiation of the slow tonic ALD muscle (Gardahaut *et al.*, 1989).

Oxidative activities appear to be similar in innervated-shortened ALD, in innervated-stretched ALD and in control ALD. In contrast, in denervated muscle, either stretched or shortened, oxidative activities are nearly undetectable. These results demonstrate that the development of these enzymatic activities requires the presence of the motor nerve.

In vitro *differentiation of presumptive slow and fast quail myoblasts in the absence of nerve*

Primary cultures were established from 9-day-old quail embryo myoblasts deriving from slow ALD and fast PLD muscle rudiments (Frémont *et al.*,1983). At this developmental stage, these two muscles are just individualized from their common primordium.

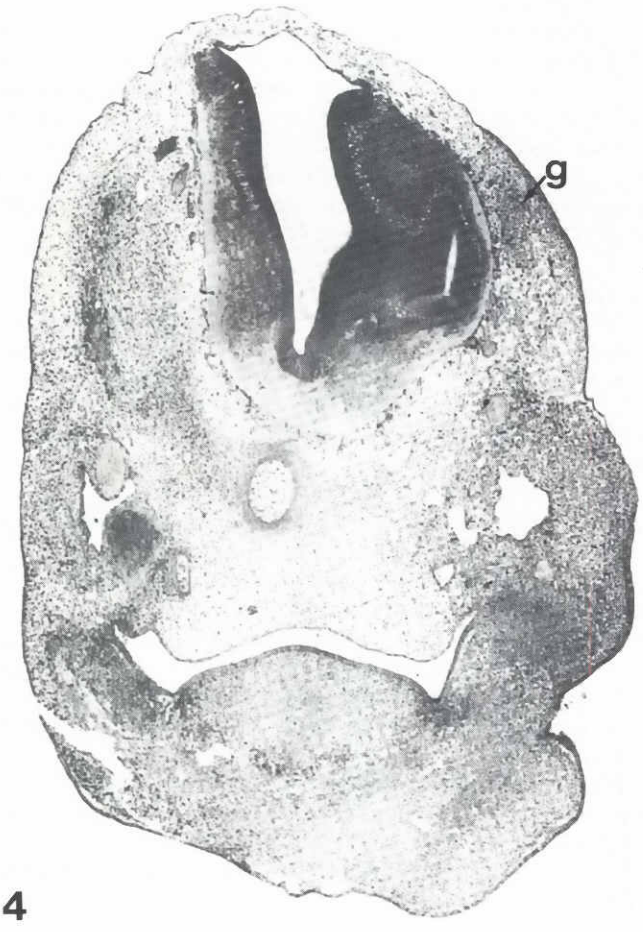

Fig. 4. Graft (g) of a 9-day quail pectoral muscle inserted between neural tube and somites 3 - 5 of a 10 somite chick embryo. *The host was fixed at day 4. Sections were hematoxylin stained. There is a unilateral enlargement of the spinal cord (x90).*

Ultrastructural features, histoenzymatic profile and accumulation of MLC isoforms were used to characterize the fiber types differentiated *in vitro* in the absence of innervation.

Aneural ALD cultures

After 3 weeks of culture without neurons, ALD-derived muscle fibers exhibit wide Z lines, numerous mitochondria, and a poorly developed sarcotubular system. On the basis of these ultrastructural features, the level of differentiation is identical to that of the muscle fibers present in 2-week-old posthatched quails. The muscle fibers stain positively for mATPase after either alkaline or acid preincubation and exhibit a high level of oxidative activity (NADH-TR), and are consequently of the slow IIIB type. All slow and fast MLC are expressed in ALD cultures but the fast LC3F appears only in trace amounts (Fig. 3A) (Frémont *et al.*, 1983; Gardahaut *et al.*, 1984).

Aneural PLD cultures

PLD-derived fibers exhibit narrow Z lines, few mitochondria, and an abundant sarcotubular system. Staining for mATPase and NADH-TR shows that these fibers are of I slow type and of IIA fast type. The accumulation of MLC in PLD cultures is quantitatively different from that occurring in ALD cultures. The fast/slow MLC ratio in PLD cultures is higher than in ALD cultures, this difference resulting mainly from the consistent accumulation of the fast LC3F in PLD cultures (Fig. 3) (Frémont *et al.*, 1983; Gardahaut *et al.*, 1984).

In conclusion, this study shows that myoblasts coming from slow and fast muscle rudiments can express *in vitro* characteristics of the slow and fast types according to their origin – ultrastructural features, enzymatic pattern and MLC accumulation – independently of motor innervation. Nevertheless, some muscle fiber types, namely IIIA in ALD cultures and II in PLD cultures, do not differentiate. In addition, the proportions of the slow and fast MLC expressed *in vitro* are respectively lower than in the ALD and PLD muscles at the end of embryonic life.

Influence of neurons on the* in vitro *differentiation of presumptive slow and fast quail myoblasts

In order to study the role played by motor innervation on muscle fiber differentiation *in vitro*, neurons from 2-day-old mouse brachial spinal cord were added to 3-week-old aneural muscle cultures; the co-cultures were grown for 2 weeks (Frémont *et al.*, 1985).

ALD co-cultures

The addition of neurons to cultures of presumptive slow myoblasts allows the occurrence of slow-tonic IIIA fibers beside the IIIB fibers that were present in aneural condition. In ALD co-cultures, the fast/slow MLC ratio is lower than in aneural cultures, this resulting mainly from an increase in the proportion of the slow MLC, LC1S and from a decrease in the proportion of the fast MLC, LC1F (Fig. 3A). These events are in agreement with the evolution of MLC proportions occurring during the *in vivo* maturation of ALD muscle (Frémont *et al.*, 1985).

These results demonstrate that the presence of neurons enhances the level of differentiation of ALD muscle fibers *in vitro* towards the slow muscle type.

PLD co-cultures

In PLD co-cultures, the presence of fibers exhibiting a histochemical profile identical with that of the slow-tonic IIIA fibers is inconsistent with the normal development of a fast muscle. In addition, the presence of neurons does not induce any increase in the fast/slow MLC ratio and even provokes a marked decrease in the fast LC3F accumulation (Fig. 3B) (Frémont *et al.*, 1985).

These data show that neurons do not enhance the differentiation of PLD fibers *in vitro* towards the fast muscle type. The effects induced by innervation could be related to the rhythm of spontaneous activity of neurons that probably occurs at a low frequency in culture. In order to test this hypothesis, experiments are now in progress and consist of imposing an activity at a fast rhythm to the neurons innervating PLD muscle fibers *in vitro*.

Influence of skeletal muscle tissue back-transplants on the proliferation of neuroepithelial cells

If it is clear that in embryonic development motor innervation plays an important role in the definitive differentiation of muscle tissue, until now no experiments have been performed to learn whether the muscle tissue can act *in ovo* on the developing spinal cord from which the motor innervation originates.

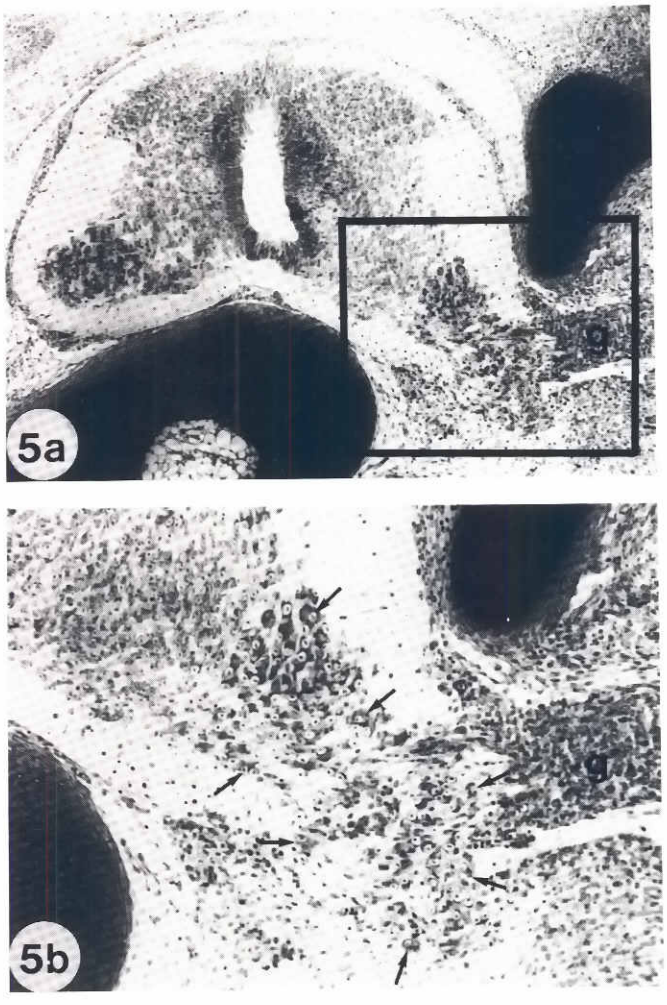

Fig. 5. Graft (g) of a 9-day quail pectoral muscle tissue cultivated for 4 weeks placed between somites 10 - 12 and the neural tube of a 11 somite chick host embryo. *The host embryo was fixed at day 9. Sections were toluidine blue stained.* **(a)** *The spinal cord has been induced to expand on the side of the graft (x70).* **(b)** *A higher magnification view of the area shown in the rectangle in (a) (x210). Motor neurons (arrows) are identified and appear more numerous on the side of the graft (g) than contralaterally.*

Back-transplantation of quail embryonic skeletal muscle

Experiments in which muscle tissue of quail embryos was back-transplanted to permit it to interact with the neural tube were undertaken. Fragments of 8- to 15-day skeletal muscle were removed from quail embryos and grafted between the neural tube and somites of 2-day chick host embryos.

Myotubes had already formed in the tissue removed for grafting at the developmental age of the donor embryos from which skeletal (*pectoralis major*, ALD, PLD) muscles were obtained. The grafts survived well in the hosts and two days after implantation had the appearance of a dense, highly cellular mass of fibromuscular tissue. At 5 days, the back-transplanted muscle became reorganized and displayed the typical morphology of skeletal muscle. In all of the grafts, examined 2-8 days following implantation, in which a close apposition between the grafted muscle and the neural tube was maintained, a unilateral enlargement of the spinal cord was observed on the side facing the graft (Fig. 4). In contrast, in those instances in which a somite had developed between the grafted muscle and the spinal cord, no spinal cord enlargement was seen. Moreover, when the grafts of skeletal muscle were placed next to the dorsal aorta instead of into the space between the somite and neural tube, enlargement of the spinal cord again failed to occur. The pattern of enlargement of the spinal cord in response to implants of skeletal muscle was very similar to that previously found for grafts of gut (Rothman *et al.*, 1987), the effect of the grafts on the neural tube being generally limited in scope to the region in which donor muscle cells were found. These observations show that muscle tissue can increase the rate of proliferation of neuroepithelial cells when these tissues are experimentally placed together.

Back-transplantation of in vitro *cultured muscle tissue*

In order to determine whether the neuroepithelium reacts similarly to *in vitro* cultured as well as *in vivo* differentiated muscle tissue, experiments were recently done in which fragments of quail skeletal muscle cultures were back-transplanted into chick host embryos. The primary cultures were established from 9-day-old quail embryo myoblasts originating from different muscle rudiments (ALD, PLD, *Pectoralis major*). After 4 weeks *in vitro*, fragments of the muscle cultures were inserted between the somites and the neural tubes of 2-day-old chick hosts. In these conditions the grafted cultures survived well in the hosts and after 2 days following surgery a dense cellular mass was found adjacent to the developing spinal cord. A unilateral enlargement of the spinal cord was obtained in response to the graft, with the pattern of enlargement resembling that found after grafts of skeletal muscle differentiated *in vivo*. Again both gray and white matter were enlarged, the growth promoting activity of skeletal muscle on the neuroepithelium thus appears to be shared by muscle tissue cultured *in vitro* (Fontaine-Perus, personal communication).

The following experiments were done to know whether the unilateral increase observed could have an influence on the neuron survival in the motor column of the spinal cord. In the few cases observed 8 days following implantation in which a close apposition between the grafts and the neural tube was obtained, an increased number of motor neurons on the operated side was noted (Fig. 5), this effect appearing to be limited to the region in which donor previously cultured muscle cells were found. A significant increase in cell number was counted between experimental and control sides in the absolute number of motor neurons in the spinal cords of chick embryos killed 8 days following the grafts.

Discussion

Our results (Toutant *et al.*, 1979), in concert with others (Butler and Cosmos, 1981; Mc Lennan, 1983; Phillips and Bennett, 1984) show that in the slow ALD and fast PLD muscles the differentiation of fiber types takes place in the first week of chick embryogenesis. By as soon as day 11, slow and fast muscle fibers become specialized with respect to native myosin isoform composition (Gardahaut *et al.*, 1988b). But the acquisition of mature muscle characteristics requires changes in the fiber type profile and in the content in isomyosins that occur during embryonic and postnatal periods (Gardahaut *et al.*, 1985, 1988b; Khaskiye *et al.*, 1987b).

The establishment of neuromuscular contacts occurs in chick

latissimus dorsii muscles between day 6 and day 12 of embryogenesis (Smith and Slater, 1983). At day 12 distinct activation patterns – phasic and tonic – were evidenced in chick motor neurons (O'Donovan, 1984). Thus it was of interest to determine whether these different patterns of motor neuron activity were able to influence the *in vivo* development of the fast and slow neuromuscular systems. Experiments with embryonic spinal cord stimulation show that the frequency pattern of nerve activity is an important factor controlling the differentiation of fast and slow features of muscle fibers during embryogenesis (Renaud *et al.*, 1978, 1983; Toutant *et al.*, 1980b, 1981b; Gardahaut *et al.*, 1983, 1985, 1988a; Fournier Le Ray *et al.*, 1986, 1989). In the PLD muscle, spinal cord stimulation imposed at day 10 induces changes in the normal PLD muscle differentiation; a change in the rhythm of motor neuron activity modifies the differentiation of the ALD muscle only when it is initiated before day 10. These results demonstrate that the plasticity of ALD muscle fibers decreases as embryonic development proceeds.

By using direct electrical stimulation of denervated muscles the role of muscle activity and of its pattern frequency in the control of certain characteristics of the slow ALD and fast PLD muscles was clearly shown during post-natal development. But it is noteworthy that the denervated slow-tonic ALD muscle exhibits less plasticity than the denervated fast-twitch PLD muscle with respect to MLC accumulation, oxidative activity and AChE molecular form distribution. This may be attributed to the intrinsic properties of the slow muscle (Khaskiye *et al.*, 1987a; Khaskiye and Renaud, 1988).

Although most of the changes induced in the denervated muscles by direct stimulation appear, in particular in the fast PLD, to be rhythm dependent, it must be underlined that some muscle characteristics behave differently. Such is the case with the CPK activity in which inhibition occurring after denervation in both ALD and PLD muscles is suppressed by direct stimulation whatever its rhythm, suggesting that this enzyme activity depends more on the amount of activity than on its frequency pattern. On the other hand, our data show that the decrease in the adult fast MLC content (LC3F) following denervation is prevented by muscle stimulation. However, since its accumulation is markedly higher after stimulation at a fast rhythm than after a slow one, it appears that the accumulation of LC3F is influenced by both the frequency and the amount of activity. Enzymatic activities, such as the LDH activity in the denervated PLD and the MDH activity in the denervated ALD, are not restored by a stimulation respectively imposed at a fast rhythm in the PLD and at slow one in the ALD. Thus it could be hypothesized that the control of muscle properties requires the involvement of factors other than muscle activity alone. In this view we have demonstrated that, in the innervated as well as in the denervated ALD muscles, a shortening results in a lack of maturation and a stretching in an enhancement of muscle differentiation. Our study also shows that the stretch can, independently of the presence of innervation, be involved in the mechanisms regulating the expression of mATPase and myosin components.

Among the other factors that may influence the post-natal development of the muscle, several authors have reported that neural trophic factors are partially responsible for maintaining certain morphologic, biochemical and physiological properties of skeletal muscle (see for instance: Jolesz and Sreter 1981; Mc Ardle, 1983; Davies *et al.*, 1985; Spector, 1985). In the present study we report that myoblasts from embryonic anterior ALD and posterior PLD *latissimus dorsii* muscles cultured *in vitro* in absence of neurons acquire characteristics of slow and fast muscle fibers according to their origin, suggesting that such myoblasts are partly preprogrammed for specific fiber type expression (Frémont *et al.*, 1983). However, the aneural condition does not allow the full expression of differentiation of slow and fast muscle fibers. For instance the slow type III A in ALD cultures and the fast type II in PLD cultures do not differentiate. In addition, in ALD and PLD cultures the proportion of slow and fast MLC is respectively lower than in the ALD and PLD muscles differentiated *in vivo*. When muscle fibers are co-cultured with neurons from spinal cord fragments of newborn mice, the differentiation of ALD muscle fibers is favored towards the slow muscle type. In contrast, the presence of innervation does not enhance the fast muscle phenotypic expression in PLD muscle fibers. The question arises whether these *in vitro* modifications of differentiation induced by innervation can be related to the slow rhythm of activity of neurons in culture (Fremont *et al.*, 1985).

Our study emphasizes that, among other factors, innervation, i.e the spinal cord motor neurons either by their presence or by their activity pattern, plays an important role in the *in vivo* and *in vitro* differentiation of muscle fibers. But until now, no experiments have established that embryonic muscles can act on the early development of the spinal cord. Experiments were done in which fragments of skeletal muscle were back-transplanted between the somites and the neural tubes of 2-day-old chick host embryos, with a subsequent unilateral increase in the number of cells in the region of the early spinal cord adjacent to the graft (Fontaine-Perus *et al.*, 1989). Embryonic skeletal muscle exhibited growth-promoting activity on the neuroepithelium of the host embryos. It is important to emphasize the fact that in these experiments the mitogenic activity of back-transplanted muscle-containing tissues is exerted at a time when the neurons (Oppenheim *et al.*, 1989) and the non-neuronal cells are being generated. When embryos in which cultured muscle fragments implanted at day 2 were examined at later development, the enlargement of the spinal cord on the operated side was extremely pronounced and the motor neuron number in the affected segment had increased (Fontaine-Perus, personal communication). These observations demonstrate that muscle-containing tissues can increase the rate of proliferation of neuroepithelial cells and modify the motor neuron proportion in the spinal cord when these tissues are experimentally placed together.

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References

ASHMORE, C.R. and DOERR, L. (1971). Postnatal development of fiber types in normal and dystrophic skeletal muscle of the chick. *Exp. Neurol. 30:* 43-446.

ASHMORE, C.R., KIKUCHI, T. and DOERR, L. (1978). Some observations on the innervation patterns of different fiber types of chick muscle. *Exp. Neurol. 58:* 272-284.

ASIEDU, S. and SHAFIQ, S.A. (1972). Actomyosin ATPase activity of the *anterior latissimus dorsi* muscle of the chicken. *Exp. Neurol. 35:* 211-213.

BARNARD, E.A., LYLES, J.M. and PIZZEY, J. A. (1982). Fiber types in chicken skeletal muscles and their changes in muscular dystrophy. *J. Physiol. (London) 331:* 333-354.

BUTLER, J. and COSMOS, E. (1981). Differentiation of the avian *Latissimus dorsi*

primordium: analysis of fiber type expression using the myosin ATPase histochemical reaction. *J. Exp. Zool. 218:* 219-232.

BUTLER, J., COSMOS, E. and BRIERLEY, J. (1982). Differentiation of muscle fiber types in aneurogenic brachial muscles of the chick embryo. *J. Exp. Zool., 224:* 65-80.

CROW, M.T. and STOCKDALE, F.E. (1986). Myosin expression and specialization among the earliest muscle fibers of the developing avian limb. *Dev. Biol. 113:* 238-254.

D'ALBIS, A., PANTALONI, C. and BECHET, J.J. (1979). An electrophoretic study of native myosin isozymes and of their subunit content. *Eur. J. Biochem. 99:* 261-272.

DAVIES H.L., HEINICKE, E.A., COOK, R.A. and KIERNAN, J.A. (1985). Partial purification from mammalian peripheral nerve of tropic factor that ameliorates atrophy for denervated muscle. *Exp. Neurol. 89:* 159-171.

FONTAINE-PERUS, J., CHANCONIE, M., LE DOUARIN, N.M., GERSHON, M. and ROTHMAN, T. (1989). Mitogenic effect of muscle on the neuroepithelium of the developing spinal cord. *Development 107:* 413-422.

FOURNIER LE RAY, C., RENAUD, D. and LE DOUARIN, G.H. (1986). Effect of spinal cord stimulation on the metabolism of developing *latissimus dorsii* muscles in chick embryo. *Cell Diff. 19:* 225-228.

FOURNIER LE RAY, C., RENAUD, D. and LE DOUARIN, G.H. (1989). Change in motor neuron activity modifies the differentiation of a slow muscle in chick embryo. *Development 106:* 295-302.

FREMONT, P.H., FOURNIER LE RAY, C. and LE DOUARIN G.H. (1983). *In vitro* differentiation in the abscence of nerve of avian myoblasts derived from slow and fast rudiments. *Cell Diff. 13, 4:* 325-340.

FREMONT, P.H., GARDAHAUT, M.F., ROUAUD, T., RENAUD, D. and LE DOUARIN, G.H. (1985). Influence of neurons on the occurence of fiber types and myosin light chains in cultured presumptive slow and fast myoblasts. *Exp. Cell Res. 160:* 230-235.

GARDAHAUT, M.-F., ROUAUD, T., RENAUD, D. and LE DOUARIN, G. (1983). Developmental change in choline acetyltransferase activity in nerve endings of *latissimus dorsii* muscles in the chick embryo: influence of chronic spinal cord stimulation. *Neurosci. Lett. 43:* 299-302.

GARDAHAUT, M.-F., FREMONT, P.H., ROUAUD, T., RENAUD, D. and LE DOUARIN, G.H. (1984). Myosin light chains synthesized by avian fast and slow myoblasts cultured in aneural conditions. *IRCS Med. Sci. 12:* 998-999.

GARDAHAUT, M.-F., ROUAUD, T., RENAUD, D. and LE DOUARIN, G. (1985). Influence of spinal cord stimulation upon myosin light chain and tropomyosin subunit expression in a fast muscle (*posterior latissimus dorsi*) of the chick embryo. *J. Musc. Res. Cell Mot.6:* 769-781.

GARDAHAUT, M.-F., KHASKIYE, A., ROUAUD, T., RENAUD, D. and LE DOUARIN, G.H. (1988a). Innervation, rythme d'activité et accumulation des chaînes légères de myosine d'un muscle rapide de poulet. *Reprod. Nutr. Dev. 28:* 773-780.

GARDAHAUT, M-.F., ROUAUD, T., RENAUD, D. and LE DOUARIN, G.H. (1988b). Developmental changes in myosin isoforms from slow and fast *latissimus dorsi* muscles in the chicken. *Differentiation 37:* 81-85.

GARDAHAUT, M.-F., ROUAUD, T.P., RENAUD, D., KHASKIYE, A. and LE DOUARIN, G.H. (1989). Role of nerve and tension in maturation of posthatching slow-tonic muscle in chicken. *Muscle Nerve 12:* 943-952.

GAUTHIER, G.F., DANA ONO, T. and HOBBS, A.W. (1984). Curare-induced transformation of myosin pattern in developing skeletal muscle fibers. *Dev. Biol. 105:* 144-154.

GINSBORG, B.L. and MACKAY, B. (1961). A histochemical demonstration of two types of motor innervation in avian skeletal muscle. In *Histochemistry of Cholinesterase. Bibl. Anat. 2:* 174-181.

HARRIS, A.J. (1981). Embryonic growth and innervation of rat skeletal muscles. I. Neural regulation of muscle fiber numbers. *Philos. Trans. R. Soc. Lond. (Biol.) 293:* 257-277.

HESS, A. (1961). Structural differences of fast and slow extrafusal muscle fibers and their nerve endings in chickens. *J. Physiol. (London) 157:* 221-231.

HOH, J.F.Y. (1979). Developmental changes in chicken skeletal myosin isoenzymes. *FEBBS Lett. 98 (2):* 267-270.

JOLESZ, F. and SRETER, F.A. (1981). Development, innervation and activity pattern-induced changes in skeletal muscle. *Ann. Rev. Physiol. 43:* 531-552.

KHASKIYE, A., TOUTANT, J.P., TOUTANT, M., RENAUD, D. and LE DOUARIN, G.H. (1980). Effects of heterotopic innervation on the development of synaptic pattern in chick embryo muscles. *Arch. Anat. Microsc. Morphol. Exp. 69:* 135-146.

KHASKIYE, A., RENAUD, D. and LE DOUARIN, G.H. (1986). Effects of denervation and direct electrical stimulation upon the post-hatching differentiation of *posterior latissimus dorsi* muscle in chicken. *Cell Diff. 18:* 27-35.

KHASKIYE, A., GARDAHAUT, M-F., FOURNIER LE RAY, C., ROUAUD, T., RENAUD, D. and LE DOUARIN, G.H. (1987a). Effects of low and high frequency patterns of stimulation on contractile properties, enzyme activities and myosin light chain accumulation in slow and fast denervated muscles of the chicken. *Pflügers Arch. 410:* 433-440.

KHASKIYE, A., RENAUD, D. and LE DOUARIN G.H. (1987b). Effects of electrical stimulation upon post-hatching development of fiber types in normally innervated fast and slow *latissimus dorsii* muscles of the chicken. *Biol. Cell. 61:* 163-170.

KHASKIYE, A. and RENAUD, D. (1988). Posthatching changes in levels and molecular forms of acetylcholinesterase in slow and fast muscles of the chicken: effects of denervation and direct electrical stimulation. *Differentiation 39:* 28-33.

LAING, N.G. and LAMB, A.H. (1983). The distribution of muscle fiber types in chick embryo wings transplanted to the pelvic region is normal. *J. Embryol. Exp. Morphol. 78:* 67-82.

Mc ARDLE J.J. (1983). Molecular aspects of the trophic influence of nerve on muscle. *Prog. Neurobiol. 21:* 135-198.

Mc LENNAN, I.S. (1983). Differentiation of muscle fiber types in the chicken hind-limb. *Dev. Biol. 97:* 222-228.

O'DONOVAN, M.J. (1984). Firing patterns and recruitment behavior of single embryonic motor neurons. *Soc. Neurosci. 10:* 915 (Abstr.).

OPPENHEIM, R.W., COLE, T. and PREVETTE, D. (1989). Early regional variations in motor neuron numbers arise by differential proliferation in the chick embryo spinal cord. *Dev. Biol. 133:* 468-474.

PHILLIPS, W.D. and BENNETT, M.R. (1984). Differentiation of fiber types in wing muscles during embryonic development: effect of neural tube removal. *Dev. Biol. 106:* 457-468.

RENAUD, D., LE DOUARIN, G.H. and KHASKIYE, A. (1978). Spinal cord stimulation in chick embryo: effects on development of the *posterior latissimus dorsi* muscle and neuromuscular junctions. *Exp. Neurol. 60:* 189-200.

RENAUD, D., GARDAHAUT, M-F., ROUAUD, T. and LE DOUARIN, G.H. (1983). Influence of chronic spinal cord stimulation upon differentiation of ß muscle fibers in a fast muscle (*posterior latissimus dorsi*) of the chick embryo. *Exp. Neurol. 80:* 157-166.

ROTHMAN, T., GERSHON, M., FONTAINE-PERUS, J., CHANCONIE, M. and LE DOUARIN, N. (1987). The effect of backtransplants of the embryonic gut wall on growth of the neural tube. *Dev. Biol. 124:* 331-346.

SMITH, M.A. and SLATER, C.R. (1983). Spatial distribution of acetylcholine receptors at developing chick neuromuscular junctions. *J. Neurocytol. 12:* 993-1005.

SOHAL, G.S. and SICKLES, D.W. (1986). Embryonic differentiation of fiber types in normal, paralyzed and aneural avian superior oblique muscle. *J. Embryol. Exp. Morphol. 96:* 79-97.

SPECTOR, S.A. (1985). Trophic effects on the contractile and histochemical properties of rat *soleus* muscle. *J. Neurosci. 5:* 2189-2196.

TOUTANT, J-P., TOUTANT, M., RENAUD, D. and LE DOUARIN, G.H. (1979). Enzymatic differentiation of muscle fiber types in embryonic *latissimus dorsii* of the chick: effects of spinal cord stimulation. *Cell Diff. 8:* 375-382.

TOUTANT, M., BOURGEOIS, J-P., TOUTANT, J-P., RENAUD, D., LE DOUARIN, G. and CHANGEUX, J-P. (1980a). Chronic stimulation of the spinal cord in developing chick embryo causes the differentiation of multiple clusters of acetylcholine receptors in the *posterior latissimus dorsi* muscle. *Dev. Biol. 76:* 384-395.

TOUTANT, J-P., TOUTANT, M., RENAUD, D. and LE DOUARIN, G.H. (1980b). Histochemical differentiation of extrafusal muscle fibers of the *anterior latissimus dorsi* in the chick. *Cell Diff. 9:* 305-314.

TOUTANT, M., TOUTANT, J-P., RENAUD, D. and LE DOUARIN, G.H. (1981a). Effects of spinal cord stimulation on the differentiation of *posterior latissimus dorsi* nerve in the chick embryo. *Exp. Neurol. 72:* 267-280.

TOUTANT, J-P., TOUTANT, M., RENAUD, D., LE DOUARIN, G. and CHANGEUX, J-P. (1981b). Effet de la stimulation médullaire chronique sur le nombre total de sites d'activité acétylcholinestérasique du muscle *posterior latissimus dorsi* de l'embryon de poulet. *C. R. Séances Acad. Sci. (Paris) (III)292:* 771-775.