Expression of myosin heavy chain isoforms in developing rat muscle spindles

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Summary. The development of muscle spindles, with respect to the expression of myosin heavy chain isoforms was studied in rat hind limbs from 17 days of gestation up to seven days after birth. Serial cross-sections were labelled with antibodies against slow tonic, slow twitch and neonatal isomyosins, myomesin, laminin and neurofilament protein.

At 17-18 days of gestation, a small population of primary myotubes expressing slow tonic myosin were identified as the earliest spindle primordia. These myotubes also expressed slow twitch and, to a lesser extent, neonatal myosin. At 19-20 days of gestation a second myotube became apparent; this staining strongly with anti-neonatal myosin. A day later this secondary myotube acquired reactivity to anti-slow tonic and anti-slow twitch myosins. By birth, a third myotube was present; this staining strongly with anti-neonatal myosin but otherwise unreactive with the other antibodies against myosin heavy chains. Three days after birth a fourth myotube, with identical reactivity to the third one, became apparent. Regional variation in the expression of isomyosins, which was present since birth in the two nuclear bag fibers was further enhanced: the nuclear bag2 staining strongly with anti-slow tonic and anti-neonatal in the equatorial region and with decreasing intensity towards the poles, whilst with anti-slow twitch the stainability was low in the equatorial and high in the polar region. The nuclear baga fiber showed a homogeneous staining: high with anti-slow tonic, moderate with anti-neonatal, and displayed stainability to anti-slow twitch myosin in the polar regions only. No regional variation was found along the chain fiber/myotube.

At seven days after birth, the pattern of reactivity was similar to that found in the adult spindles, except for the bag1 fiber which still expressed neonatal myosin.

We show that slow tonic myosin is expressed from early development and it is a reliable marker of developing bag fibers. We suggest that muscle spindles are formed from special cell lineages of which the primary generation myotubes expressing slow tonic myosin represent the primordium of muscle spindles.

Introduction

Muscle spindles are encapsulated sensory organs composed of intrafusal fibers which are specialized muscle fibers innervated by both sensory and motor neurons. Generally three different types of intrafusal fibers can be distinguished in mammalian muscle spindles: nuclear bag1, nuclear bag2 and nuclear chain fibers (for review see Barker and Banks 1986). Intrafusal fibers are, in general, separated into slow twitch (type 1) fibers and fast twitch (type 2, with subtypes type 2A and 2B) fibers. One basic feature which largely determines the properties of these fibers is the type of myosin expressed. Type 1 fibers contain slow twitch myosin, and type 2 fibers different isoforms of fast twitch myosins (Billeter et al. 1981; Pierobon-Bormioli et al. 1981; Schiaffino et al. 1989; Termin et al. 1989). The expression of myosin isoforms in intrafusal fibers is, however, more complex. The unique expression of slow tonic, embryonic and neonatal myosin heavy chains (MHCs) is restricted to the intrafusal fibers, in limb muscles of mammals (Pierobon-Bormioli et al. 1980; Rowleson et al. 1985; Maier et al. 1988; Kucera and Walro 1988; Pedrosa et al. 1989). In rat spindles, the bag fibers mainly contain slow forms of MHC whereas the chain fibers mainly have fast isoforms. Furthermore, expression of the different isomyosins varies along the length of the fibers (Pedrosa et al. 1989).

Development of muscle spindles in the primordia of the rat hind limb muscles starting at the late fetal stages continues till the end of the first month after birth (Zelená 1957). In electron micrographs, the first signs of muscle spindles are the formation of simple primary sensory contacts between nerve terminals and single myo-
tubes indistinguishable from extrafusal myotubes of comparable size (Landon 1972; Milburn 1973; Kucera et al. 1989b). The nuclear bag2 fiber is the first to arise, followed by the nuclear bag1 and each of the nuclear chain fibers, so that four days after birth the full complement of four intrafusal fibers is present (Milburn 1973).

Sequential transition in the expression of MHC isoforms occurs in extrafusal fibers during rat muscle development (Whalen et al. 1981; Butler-Browne and Whalen 1984; Whalen 1985; Dhoot 1985, 1986; Narusawa et al. 1987). In the early developmental stages, the first myotubes to be formed (primary generation) contain embryonic and slow MHCs followed by either fetal/neonatal and slow MHC or fetal/neonatal and fast MHC or fetal/neonatal, slow and fast MHC. The secondary generation of fibers express embryonic MHC followed by fetal/neonatal MHC and then either slow or fast MHCs. The question as to whether sequential transitions occur during the development of muscle spindles has been less explored, but some evidence exists. Intrafusal fibers in spindles of newborn and adult rat differ in their reactivity to polyclonal antiserum against MHC (te Kronnie et al. 1982). Thornell et al. (1988) using immunocytochemistry showed that heterogeneity existed within the primary generation of myotubes in mammals; primary myotubes that contain slow tonic MHC very early in the development being destined to become nuclear bag fibers. They also observed that both the primary and secondary generation of myotubes of spindle fibers expressed slow tonic MHC. Kucera and Walro (1989) using two monoclonal antibodies, one against slow tonic myosin and one against neonatal and fast myosin, have recently reported that bag fibers in neonatal rats expressed immature myosin patterns but chain fibers did not, and that the adult pattern of reactivity with the two antibodies became apparent by the fourth postnatal day.

In order to further understand the development of muscle spindles with respect to the expression of MHC, we have used immunocytochemical methods, and a battery of antibodies against different isomyosin heavy chains in hind limb muscles of rats from 17 days of gestation up to seven days after birth. Our results further increase our knowledge on early spindle development and provide an overall view of the expression of MHC isoforms and the subsequent changes occurring in each fiber type during maturation.

A preliminary summary of part of the data presented here has been published previously (Thornell et al. 1989).

**Materials and methods**

**Material.** In the present study, Sprague-Dawley rats of the following ages were used: 17-, 18-, 19-, 20- and 21-days gestation, and 1-, 3- and 7-days old. The age of the fetal (F) material was taken to be the number of days following the time of conception which was assumed to coincide with the appearance of the vaginal plug. Gestation lasted approximately 21 days. The age of the postnatal rats was determined from the moment of birth. These age assessments are accurate to approximately 12 h.

Sections of the lower hind legs (knee to ankle) or of the soleus, tibialis anterior (TA) and extensor digitorum longus (EDL) muscles were rapidly frozen in propane chilled in liquid nitrogen. Serial cross sections were cut in Zeiss or Reichert Jung cryostats at −19° C. At regular intervals, three sections 9 μm thick were taken and stained to demonstrate myofibrillar ATPase activity after pre-incubation at pH 9.4, 4.6 and 4.3 (Dubowitz and Brooke 1973), and the remaining sections, 4–5 μm thick, were processed for immunocytochemistry.

**Antibodies and labelling.** Previously characterized mono- (mAb) and polyclonal antibodies against myosin heavy chains (MHC) were used. The mAb Ald 19 was prepared against chicken anterior lattissimus dorsi (ALD) myosin (Sawchak et al. 1985) and it shows strong affinity to slow tonic myosin when used in low dilutions (Thornell et al. 1989; Pedrosa et al. 1989). In human fetal muscle, mAb ALD 19 shows weak reactivity with an epitope present in primary generation fibers, whereas in adult muscle, weak reactivity is seen in type II fibers (Thornell et al. 1988, 1989). The mAb 9812 against slow twitch MHC (Kilby and Dhoot 1988), and a polyclonal antiserum NN5 against neonatal MHC (Butler-Browne and Whalen 1984) were also used. The reactivity of adult rat intrafusal fibers to these antibodies has been documented previously (Pedrosa et al. 1989).

In addition, antibodies against 1) myomesin, a protein of Mr 185000 localized in the myofibrillar M band (Grove et al. 1984), 2) laminin, present in basement membranes (Sanes and Cheney 1982) and 3) neurofilament protein (subunit 68 kD), present in intermediate filaments of neuronal tissue (Hacker et al. 1985), were also used.

The mAb ALD 19 was a generous gift from Dr. D.A. Fischman, mAb 9812 from Dr. G.A. Dhoot, anti-myomesin from Dr. B.K. Grove, and the polyclonal antiserum NN5 from Dr. G.S. Butler-Browne. Anti-laminin was purchased from E-4 labs inc, San Mateo, CA, USA, and anti-neurofilament Dako-NF, was purchased from Dakopatts, Copenhagen, Denmark. Standard indirect peroxidase-antiperoxidase (PAP) and indirect immunofluorescence techniques were used. Secondary FITC antibodies and PAP were obtained from Dakopatts, Copenhagen, Denmark. The sections were examined in a Leitz Dialux microscope or in an Olympus Vanox microscope equipped with epifluorescence.

**Survey.** Several specimens were sectioned from each rat. From each age group, two to four optimally cross sectioned specimens were further serially sectioned and analyzed in detail. In the fetal rats, a series of at least 20–30 cross-sections of the entire hind leg were surveyed. A minimum of 20 and up to 100 serial sections of the postnatal specimens, the soleus, EDL and TA muscle, were analyzed. For any given age, 8–20 muscle spindles were studied.

In each muscle spindle, three regions (A, B and C) defined according to Barker and Banks (1986) were considered. The A region, corresponded to the equatorial and juxtaequatorial regions containing the periaxial space. The B region extended from the end of the periaxial space to the end of the capsule, and the C region, corresponded to the extracapsular part of the muscle spindle.

Distinction of intrafusal fiber types was based upon fiber length, diameter and morphology of the nuclei in the equatorial region. The antibody against myomesin was used as a marker to determine the number of fibers, because it stains myofibrils in all myotubes and myofibers irrespective of age (Grove et al. 1984, 1988). Correlation to the pattern of immunoreactivity of the adult rat intrafusal fibers (Pedrosa et al. 1989) was a further criterion.

**Results.**

**17F–18F**

At 17 to 18 days of gestation, the primordia of tibialis anterior (TA), extensor digitorum longus (EDL) and so-