Treatment with \( \beta \) bungarotoxin blocks muscle spindle formation in fetal rats

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Summary

Sensory and motor fibers of peripheral nerves were irreversibly destroyed in fetal rats by administering \( \beta \) bungarotoxin (BTX) on embryonic day 16 or 17, after assembly of primary myotubes, but before the formation of muscle spindles. Soleus muscles of toxin-treated fetuses and their untreated littermates were removed just prior to birth and were examined by light microscopy of serial transverse sections for the presence of spindles and immunocytochemical expression of several isoforms of myosin heavy chains (MHC). Untreated muscles exhibited numerous spindles that were innervated by branches of intramuscular nerves and contained muscle fibers expressing a slow-tonic MHC isoform characteristic of the intrafusal but not extrafusal fibers. Toxin-treated muscles were devoid of intramuscular nerve bundles and perineurial structures. Encapsulations of muscle fibers resembling spindles were absent and no myotubes expressed the slow-tonic MHC isoform associated with intrafusal fibers in \( \beta \) BTX-treated muscles. Thus, the assembly of muscle spindles, formation of the spindle capsule, and transformation of undifferentiated myotubes into the intrafusal fibers that contain spindle-specific myosin isoforms all depend on the presence of innervation in prenatal rat muscles.

Key words: myosin heavy chains, intrafusal muscle fibers, muscle differentiation, nerve dependence, fetal rat, \( \beta \) bungarotoxin.

Introduction

Intrafusal fibers of muscle spindles in rat hindlimbs differ from extrafusal muscle fibers in size, structure and function. Intrafusal fibers also express several isoforms of myosin or myosin heavy chains (MHC) that are not expressed by extrafusal fibers of the same muscle (Pierobon-Bormioli et al. 1980; Pedrosa et al. 1989). These isoforms include a slow-tonic MHC similar to that contained in the chicken anterior latissimus dorsi muscle, as well as embryonic and neonatal MHCs that persist in the intrafusal but not extrafusal fibers into adulthood (Maier et al. 1988; Kucera and Walro, 1988, 1989a). Developing intrafusal fibers of the rat soleus muscle begin to express the spindle-specific MHCs shortly before birth (Kucera and Walro, 1990).

Innervation is not essential for the assembly and initial differentiation of extrafusal muscle fibers in the rat. Hindlimb muscles of fetal rats experimentally deprived of nerve supply contain both the primary and secondary myotubes, albeit in diminished numbers (Harris, 1981; Ross et al. 1987; Condon et al. 1989). Moreover, several different isoforms of MHC are expressed by these extrafusal myotubes in the absence of innervation (Harris et al. 1989; Condon et al. 1989). However, whether the genesis of MHC isoforms in developing intrafusal fibers is also intrinsic to the muscle fibers themselves and independent of innervation is unclear. The aim of the present study was to examine whether spindles form and myotubes express the slow-tonic MHC isoform characteristic of intrafusal fibers in developing soleus muscles deprived of sensory and motor innervation prior to the onset of spindle assembly. Removal of innervation was achieved by treating fetal rats with \( \beta \) bungarotoxin (BTX), a neurotoxin that binds to and irreversibly destroys peripheral nerves (McCaig et al. 1987).

Materials and methods

Animals

Five timed pregnant Sprague-Dawley rats (the day of impregnation was considered as embryonic day zero) anesthetized with sodium pentobarbital (35 mg/100 g b.wt.) underwent laparotomy on embryonic day 16 or 17 (E16 or E17) under sterile conditions. Two fetuses of each female were injected intraperitoneally with 1 \( \mu \)g of \( \beta \) bungarotoxin (Sigma) dissolved in 1 \( \mu \)l of sterile physiological saline using a Hamilton syringe (McCaig et al. 1987). The location of treated fetuses in uterine horns was marked with a nonresorbable suture, the abdominal incision was sutured, and the rats were allowed to survive for 4 or 5 days. Treated mothers were anesthetized on E21, the uterine horns were reexposed and soleus muscles were excised from the treated fetuses and their untreated (control) littermates. Control fetuses exhibited limb movements in response to electrical stimulation of the snout (100 V, 10 Hz, 0.5 msec), whereas fetuses injected with \( \beta \) BTX did not (McCaig et al. 1987).
**Tissue processing**

The right lower leg of toxin-treated or untreated fetal rats was quenched in isopentane cooled to $-160\,^\circ\text{C}$ with liquid nitrogen, and cut transversely into serial sections of 8 $\mu$m thickness from the ankle to the knee in a cryostat. The sections were reacted with one of four monoclonal antibodies on an alternating basis. The antibodies were mouse IgGs known to bind to different isofoms of MHCs present in mammalian intrafusal and extrafusal fibers (Kucera and Walro, 1989a). Two antibodies were raised against avian (chicken) muscles, one (ALD58 or ATO) against the slow-tonic anterior latissimus dorsi and the other (MF30 or ANT) against the predominantly fast-twitch pectoralis major muscle (Baden et al. 1982). Two antibodies were raised against mammalian muscles, one (NOQ7.5-4D or MST) against human slow-twitch fibers and the other (WBMHC-f or MFT) against rabbit fast-twitch muscle fibers (Draeger et al. 1987; Ecb-Prince et al. 1989).

Binding of the primary antibody was demonstrated by an immunoperoxidase reaction utilizing the ABC (avidin–biotin complex) method (Vectastain P4002 or P6102 kit, Vector Labs., Burlingame Calif.) and a substrate reagent containing diaminobenzidine and hydrogen peroxide, as detailed previously (Kucera and Walro, 1988, 1989a). Control and toxin-treated muscles were processed for MHC immunochemistry concurrently.

The ATO antibody (diluted 1:1) binds to the slow-tonic MHC isofóm present in bag and bag$_2$ intrafusal fibers, but not in extrafusal fibers of developing muscles. The ANT antibody (1:40) binds to the neonatal-twitch MHC present in both intrafusal and extrafusal myotubes. The MST (1:2) and MFT (1:3) antibodies bind to the primary and secondary myotubes of developing rat muscles, respectively (Kucera and Walro, 1990).

The left lower leg of toxin-treated or control rats was fixed by immersion in buffered 2 % glutaraldehyde, treated with 1 % osmium tetroxide and embedded into plastic. Serial transverse sections of 1 $\mu$m thickness were cut through entire soleus muscles on a LKB Nova ultramicrotome and stained with toluidine blue.

**Identification of structures**

The developing soleus muscle was identified by locating its origin on the fibula and its insertion into the Achilles tendon. This muscle also contained a high density of large diameter myotubes binding the MST antibody as opposed to other muscles of the crus (Kucera and Walro, 1990). Primary and secondary myotubes were distinguished based on their relative size and mutual position (Harris, 1981). Developing muscle spindles were recognized as encapsulations containing one or more muscle fibers, which bound the ATO antibody in sections processed for MHC immunochemistry (Kucera and Walro, 1990).

**Results**

A total of 30 soleus muscles were removed at embryonic day 21 (E21) from ten $\beta$ BTX-treated fetal rats and five untreated (control) littermates. The muscle specimens were examined for the presence of innervation, encapsulations of small diameter fibers and expression of several MHC isofoms by extrafusal and intrafusal fibers (Fig. 1A and 2).

Seven fetal rats were given $\beta$ BTX on E16. At this stage of development the soleus muscle contains all the primary, but no secondary myotubes, and there are no ultrastructurally identifiable neuromuscular junctions (Harris et al. 1989; Kucera et al. 1989). Three fetal rats were given $\beta$ BTX on E17 to allow the recently assembled primary myotubes to develop for one additional day prior to the toxin treatment. There are no spindles in the soleus muscle on E17 (Kucera et al. 1989). Results did not differ between E21 muscles treated with $\beta$ BTX on E16 or E17, thus data from all toxin-treated muscles were pooled.

**Soleus muscles**

Every control E21 muscle contained intramuscular nerve bundles that were readily identified in plastic sections stained with toluidine blue. The nerve bundles originated from the muscle nerve that entered the muscle belly in its proximal third, and contained unmyelinated nerve fibers. No peripheral nerves or remnants of nerves were observed in muscles of the lower leg of the $\beta$ BTX-treated rats, thus the soleus muscles were aneural.

Two types of myotubes were present in control E21 muscles. Primary myotubes were larger and expressed the slow-twitch (MST) MHC whereas the secondary myotubes were smaller and expressed the fast-twitch (MFT) MHC, or both MFT and MST (Kucera and Walro, 1990). Both the primary and secondary myotubes expressed the neonatal-twitch (ANT) antibody and none expressed the slow-tonic (ATO) antibody (Kucera and Walro, 1990). Toxin-treated muscles were thinner and shorter by 30–40 % than their control counterparts (Fig. 1A and B). However, primary and secondary myotubes were present in the aneural muscles, and they bound the same MHC antibodies as their counterparts in the control muscles (Fig. 2C and D). The large MST-binding primary myotubes predominated in both the treated and control soleus muscles. Occasionally, myotubes staining darkly with toluidine blue (presumed degenerating) were visible in toxin-treated but not in control muscles.

**Muscle spindles**

One or more spindles were readily identified in most cross-sections of the plastic-embedded or frozen control muscles. The ease with which spindles could be located in controls reflects the relatively high density of 18 to 20 spindles in the normal soleus muscles at E21 (Kucera et al. 1989). Spindles in sections of E21 control muscles stained with toluidine blue consisted of two intrafusal fibers surrounded by a distinct capsule (Fig. 1C). Branches of intramuscular nerves innervated the nascent intrafusal fibers. In contrast, no spindles or muscle fibers enveloped by capsule-like sheaths were visible in any of the toxin-treated muscles (Fig. 1D). Moreover, no structures that could be interpreted as degenerating spindles or spindle remnants were encountered in sections of the aneural E21 muscles stained with toluidine blue.

**Intrafusal fibers**

All intrafusal bundles of control E21 soleus muscles contained one relatively long (bag$_1$) and one relatively...
Spindle formation in aneural muscles

Fig. 1. Comparison of untreated (n) and β BTX-treated (tx) E21 soleus muscles in staining with toluidine blue. The control muscle contains two spindles marked with arrows (A). One of the spindles (s) is shown at a higher magnification (C). The toxin-treated muscle is devoid of encapsulated muscle fibers (B,D). Bars=50 μm (A,B) and 20 μm (C,D).

short (bag₁) myotube (Kucera et al. 1989). The bag fibers contained accumulations of central myonuclei near the fiber midportion at the site of the future equatorial region. All bag₂ and rare bag₁ fibers exhibited strong ATO reactivity at this stage of development (Fig. 2B). Reactivity to ATO was limited to the
Fig. 2. Sections of β BTX treated (tx) and untreated (n) E21 soleus muscles immunostained with the slow-tonic ATO (A,B), slow-twitch MST (C) and fast-twitch MFT (D) MHC antibodies. The untreated muscle contains scattered myotubes (bag2 intrafusal fibers of spindle (s) formations) that strongly bind the slow-tonic MHC antibody (B) whereas the toxin-treated muscle is devoid of such myotubes (A). The primary myotubes (p) of the toxin-treated muscle bind the slow-twitch MHC antibody, whereas fast secondary (fs) myotubes bind the fast-twitch MHC antibody and mixed secondary (ms) myotubes bind both the slow-twitch and fast-twitch MHC antibodies. Bars=50 μm (A,B) and 20 μm (C,D).
periphery of the fibers at the equator of developing intrafusal fibers.

No muscle fibers with either the morphological or immunocytochemical characteristics of the intrafusal fibers were observed in any of the toxin-treated E21 soleus muscles (Fig. 2A). No fibers contained localized clusters of central myonuclei characteristic of the equatorial region of intrafusal fibers. No muscle fibers of toxin-treated muscles bound ATO comparable to that of bag2 or bagi fibers of spindles in control E21 muscles even when the toxin-treated and control specimens were immunoprocessed jointly.

**Discussion**

A single injection of β BTX administered to fetal rats on E16 or E17 rapidly and irreversibly destroys both sensory and motor nerve fibers in all peripheral nerves (Harris, 1981; McCaig et al. 1987). No regenerating nerve fibers reach the hindlimbs during the remainder of the gestation period after a single β BTX injection at E16 or E17 (Ross et al. 1987). Indeed, limbs of the toxin-treated fetuses were paralyzed, and no intramuscular nerve bundles were visible in β BTX-treated soleus muscle by light microscopy at E21.

**Soleus muscles**

The toxin-treated muscles were smaller and shorter than muscles from untreated littermates, although they maintained their position relative to other leg muscles. The small size reflected the mild deficiency in the complements of primary and secondary myotubes resulting from removal of muscle innervation at E16 or E17 (Ross et al. 1987). The shorter length of toxin-treated E21 muscle might reflect retardation of muscle and/or bone growth in length in the absence of muscle contractile activity.

The aneural muscles contained both primary and secondary myotubes. The primary myotubes bound MST, as is characteristic for the developing rat soleus muscle (Dhoot, 1986). The smaller secondary myotubes bound either MFT only or both MFT and MST, analogous to the fast secondary and mixed secondary myotubes of normal E21 muscles (Kucera and Walro, 1990). The slow-twitch and fast-twitch MHC isoforms were expressed even though the soleus muscles were treated with β BTX prior to the establishment of the neuromuscular junctions, and prior to the assembly of secondary myotubes (Kucera et al. 1989; Harris et al. 1989). Thus, innervation is not essential for the initial expression of different MHCs by primary and secondary intrafusal myotubes. A similar conclusion was reached by Condon et al. (1989).

**Muscle spindles**

Spindles of the rat soleus muscle begin to form on E18 (Landon, 1972; Milburn, 1973). Nascent spindles can be recognized in the early E18 muscles as encapsulations containing one primary myotube (future bag2 intrafusal fiber) and a cluster of associated nerve terminals (Kucera et al. 1989). Prenatal development of a rat spindle includes three processes: encapsulation of an already assembled primary myotube at E18, assembly of an additional, secondary myotube from mononuclear cells enclosed within the capsular walls on E19–E20, and transformation of the encapsulated myotubes into the bag2 and bag1 intrafusal fibers by E21 (Kucera et al. 1989).

Rats were administered β BTX on E16 or E17, after the assembly of all primary myotubes (presumably including the future bag2 fiber), but before the onset of formation of spindles. The absence of encapsulated myotubes in toxin-treated E21 muscles suggests that innervation is essential for the process whereby some of the primary myotubes are encapsulated and the formation of spindles begins in E18 soleus muscles. The dependence of the capsule formation on innervation may reflect the origin of the capsular envelopes from the perineurium of peripheral nerves (Low, 1976). The toxin-treated muscle contained neither nerve fibers nor perineurial sheaths. The toxin has no direct action on the cells of the perineurium (McCaig et al. 1987); hence, the absence of the spindle capsules in the toxin-treated muscle reflects the dependence of the perineurial sheath development on nerve fibers.

Zelena (1957) noticed a rapid dissolution of already formed spindle capsules and intrafusal bundles following sciatic nerve section at E19 or E20. Thus, nerve fibers not only initiate the development of spindles by mediating the formation of the spindle capsule on E18, but are also essential for the persistence of the capsular sheaths enveloping the recently assembled intrafusal bundles during the late gestational period in rats.

**Intrafusal fibers**

 Destruction of nerve fibers resulting from the administration of β BTX also prevented the transformation of some of the already assembled primary myotubes into the bag2 intrafusal fibers, and the assembly of the bag1 fiber from precursor myoblasts. The toxin-treated E21 muscles contained neither muscle fibers with central aggregates of myonuclei nor fibers expressing ATO. In contrast, equatorial clusters of myonuclei and binding of ATO are features of all bag2 and some bag1 fibers in normal E19–E21 soleus muscles (Kucera and Walro, 1990). Zelena (1957) also observed that rat muscles denervated by sciatic nerve section on E19 or E20 lack intrafusal fibers containing central clusters of myonuclei. Thus, innervation is essential for the emergence of structural features characteristic of the intrafusal fibers. In particular, the expression of the slow-tonic MHC isoform in bag2 and bag1 fibers, and by implication a slow speed of contraction of these two intrafusal fiber types, may be a direct result of the action of nerve fibers on undifferentiated myotubes contained within nascent spindle encapsulations.

Both sensory and motor axons are present in the vicinity of assembling spindles in E18 soleus muscles (Kucera et al. 1989), and both kinds of axon are prevented from contacting muscle fibers by β BTX treatment. However, several lines of evidence suggest...
that afferents rather than efferents play the dominant role in the formation of spindles. First, afferents contact bag₂ or bag₁ fibers prior to the expression of slow-tonic MHC by intrafusal fibers, whereas efferents innervate intrafusal fibers after the expression of slow-tonic MHC (Kucera et al. 1989; Kucera and Walro, 1990). Second, the spindle capsule and the equatorial myonuclear clusters in intrafusal fibers persist after neonatal deafferentation (Zelená and Soukup, 1973), but disintegrate after neonatal deafferentation (Kucera and Walro, 1988). Third, deafferentation but not deafferentation abolishes ATO expression in the bag₂ and bag₁ fibers of neonatal spindles of the rat soleus muscle (Kucera and Walro, 1988). The mechanism for the ontogenetic effect of afferents might involve a release of a neurotrophic substance capable of influencing the gene expression in the equatorial myonuclear clusters subjacent to the sensory endings (Zelená, 1976; Kucera and Walro, 1989b).

The absence of ATO-reactive muscle fibers in aneural muscles did not result from selective degeneration of myotubes destined to express ATO. The toxin has no direct effect on myoblasts or myotubes (Hirokawa, 1978). Rather, β BTX acts indirectly on myotubes by the removal of innervation through a presynaptic effect (Abe et al. 1977). Delayed degeneration of primary myotubes occurs in β BTX-treated muscles, but its occurrence reflects the lack of motor innervation rather than a direct action of the toxin on the myotubes (Ross et al. 1987).

Whether the precursors of intrafusal fibers represent a population of mononuclear cells and/or myoblasts intrinsically different from those fusing and differentiating into extrafusal fibers is not known (Thornell et al. 1988). The number of soleus spindles is highly constant, and is less than the number of afferent branches in the developing soleus muscle (Kucera et al. 1989). Thus, a specific type of myotube might be essential for the formation of spindles (Kucera et al. 1989). In addition, the different types of avian intrafusal fibers originate from several lines of clonally different myotubes (Stockdale and Miller, 1987) and the precursors of intrafusal fibers might represent yet another clone of myoblasts. The β BTX-treated muscles contained no fibers reactive to ATO. Thus, rat soleus muscles do not contain a set of myotubes programmed to express ATO in the absence of innervation, at least to the extent this MHC isoform is expressed by control intrafusal fibers innervated by afferents. Absence of ATO reactivity in aneural toxin-treated muscles suggests that intrafusal fibers originate from the same precursor muscle cells as do the extrafusal fibers, and that the nerve fibers (afferents) mediate the transformation of primary myotubes into bag₁ fibers. If so, the number of spindles in a muscle may then correspond to the number of primary afferent neurons innervating the muscle.

The present study indicates that intrafusal and extrafusal muscle fibers differ fundamentally in their dependence on innervation during prenatal development. Whereas the initial MHC differentiation of extrafusal fiber types in the developing rat soleus muscle is nerve-independent (Condon et al. 1989), the differentiation of myotubes into the ATO-containing bag₂ and bag₁ intrafusal fibers and the formation of the spindle capsule depend upon innervation.

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References


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