The Morphogenetic Influence of Innervation on the Ontogenetic Development of Muscle-spindles

by J. ZELENA

From the Institute of Physiology, Czechoslovak Academy of Sciences, Prague

WITH TWO PLATES

INTRODUCTION

The influence of innervation on the differentiation of peripheral structures during ontogenesis has been studied mainly in skeletal muscle.

It has been shown in a number of experimental studies on amphibians that the morphological differentiation of the whole extremity as well as its muscular tissue takes place normally after removal of the medullary plate at the gastrula or neurula stage, although the limb developing without innervation is smaller and its musculature is atrophic (Harrison, 1903, 1904; Hooker, 1911; Hamburger, 1928). In the chick transplantation of the limb-bud into the coelom cavity or on to the chorioallantois leads in the first phase to muscular differentiation despite the fact that the limb is not innervated (Hunt, 1932; Hamburger & Waugh, 1940; Eastlick, 1943; Eastlick & Wortham, 1947). Degeneration and sarcoclysis follow the initial differentiation at a very early stage.

Similarly, an histological analysis of pathological material from human and animal embryos indicates that the differentiation of non-innervated muscular tissue takes place; the developing musculature, however, succumbs at a very early stage to degeneration and is practically replaced by adipose tissue (Weber, 1851; Anders, 1921).

Morphological differentiation of muscle-fibres in amphibians evidently takes place independently of the nervous system and may attain maturity, while in birds and mammals only the initial phase of the development of myoblasts and myotubes is possible. Further differentiation is not realized on account of rapid degeneration. An increased dependence of the histogenesis of musculature on the innervation is to be observed in these phylogenetically higher forms. The way in which the influence of the nervous system on morphogenesis of muscle changes during ontogenesis is not, however, known. This problem might be

---

1 Author's address: Československá Akademie věd, fysiologický ústav, Praha-Dejvice, Na cvičišti č. 2, Czechoslovakia.

elucidated by denervation studies during the later stages of muscular development.

It is possible to recognize three stages in the morphogenesis of muscular tissue: the myoblastic stage, the myotubal stage, and the stage of the mature muscular fibres (Tello, 1922; Studitskij & Striganova, 1951). The myoblastic stage is characterized by the production of elongated pointed myoblasts with oval nuclei from non-differentiated mesodermal cells. The fusion of myoblasts, together with the proliferation of nuclei and the differentiation of myofibrils at the periphery, characterize the myotubal stage. Further stages of development continue in either of two ways. In the majority of myotubes the nuclei migrate to the periphery, myofibrils fill up the space inside the myotube, and a mature muscle-fibre develops. A different course of histogenesis is observed in a smaller number of myotubes which become a part of neuromuscular spindles: nuclei do not migrate towards the fibre surface, but divide intensively, forming a nuclear bag which is a characteristic structure of the intrafusal muscle-fibres, i.e. fibres of the muscle-spindle.

The normal development of a muscle-spindle and its components—intrafusal fibres, nerve-endings, and connective tissue capsule—has been described in birds (Tello, 1922) and in several mammals (Sutton, 1915; Cuajunco, 1927a, b; Kalugina, 1956). There is a marked temporal coincidence between the appearance of primitive nerve-endings and the nuclear proliferation in muscle-fibres surrounded by this network. It is not at all clear, however, whether and to what extent the nervous system influences the specific differentiation of intrafusal fibres.

In this report the development of intrafusal muscle-fibres and their connective tissue capsule was studied in normal and denervated muscle with the aim of elucidating the question whether it is dependent on the influence of the nervous system, or whether the differentiation can take place even at a relatively late stage of development independently of innervation.

MATERIAL AND METHODS

Experiments were performed on rat and rabbit foetuses and on new-born and 20-day-old rats. The stage of differentiation of muscle-spindles in rats, rabbits, and guinea-pigs at birth was determined in a preliminary study. The majority of experiments was done on the rat because muscle-spindles begin to develop very late in this animal and an intra-uterine operation is more easily performed shortly before birth. The development of normal muscle-spindles was observed in the rat from the 17th day of intra-uterine life, i.e. 5 days before birth, till the 30th day after birth in calf-muscles of the hind extremity. Muscles were impregnated in frozen sections (Gross-Bielschovsky) and in toto (Cajal V). After embedding in celloidin-paraffin (Péterfi’s technique), partial series of calf-muscles were obtained, stained with Harris’s haematoxylin-eosin, Heidenhain’s haematoxylin, Azan, and a combination of the last two methods.
The development of muscle-spindles has been determined in muscles of the thorax and the forelimb of rats (Kalugina, 1956). The course of spindle development in the calf-muscles is described in detail for two reasons: (1) a slower course was found than the spindles of the thorax, (2) Kalugina primarily observed the development of innervation; a description of cytological details seems to be more important if comparison between the development of muscle-spindles in normal and denervated tissue is to be made.

The sciatic nerve was sectioned in the experimental series in the middle of the thigh in rat foetuses on the 19th–20th day of intra-uterine life, i.e. 60–72 hours before birth, on the first day after birth, and in 20-day-old rats. Material was taken 3, 4, 5, 7, and 10 days after the intra-uterine operation, and after 5 and 10 days in the remaining two series. In rabbit foetuses the sciatic nerve was sectioned on the 21st day of intra-uterine development, i.e. 7 days before birth, but experimental material was obtained only in two cases.

Neurotomy in foetuses was performed under ether anaesthesia of the mother on the exact date after insemination. After laparotomy the uterus was exposed and a purse string suture, which included the foetal membranes, was made above the right hind limb of the foetus (usually in the horn of the uterus). Under microscope control the wall of the uterus was incised and the hind limb exposed. The sciatic nerve was cut and a part was excised in order to prevent regeneration. Bleeding was stopped with the aid of fibrin foam. The wound and the foetal membranes were sutured in the rabbit foetuses only. The exposed limb was slipped back into the uterus and the purse-string suture tightened. The procedure was performed on 2–3 foetuses in each mother. Following this operation the rats were usually able to deliver normally and suckle young.

In the new-born and 20-day-old rats n. ischiadicus was sectioned close to the hip-joint and a ligature was applied to the central stump.

RESULTS

A. Development of neuromuscular spindles in the calf-muscles of the rat

On the 17th and 18th day of intra-uterine development a nervous network without terminal endings is present in the calf-muscles of the foetus. Myotubes with indistinct cross-striation do not yet exhibit any individual differences (Plate 2, fig. D). By the 19th–20th day there are to be seen in some of the myotubes zones of densely packed nuclei in the vicinity of nerve-branching arranged along the longitudinal axis of the myotube. Elongated nuclei are placed in rows closer together than in the normal myotube. This represents the beginning of spindle differentiation. The formation of spindles does not, of course, commence abruptly and does not progress quite uniformly even in the same muscle.

Immediately before birth there is further nuclear division in the future equatorial zone of the spindle. In the vicinity of this zone fibroblasts appear in the spaces in-between muscle-fibres. In most cases nerve-fibres advance towards
the future polar region of the spindle, adopt a parallel course along one or both sides of the equatorial zone, and give off fine branches to it. 

**The first day after birth** the equatorial zone in the rat is clearly differentiated and is approximately 40 \( \mu \) long and 15 \( \mu \) wide, including the differentiating capsule. On longitudinal section 1–2 intrafusal fibres with a course along the axis of the spindle may be observed. The fibres in the equatorial zone are packed with nuclei, which are strongly basophil, mostly polygonal though sometimes oval, and placed in one or two rows close to each other (Plate 2, fig. E). The intrafusal fibres are dilated in the equatorial zone by this accumulation of nuclei, which quite displaces the protoplasm. A capsule of connective tissue containing fibroblasts, placed closely one after the other, is formed around the intrafusal fibres. It is possible to observe fine reticular fibrils, stained by aniline blue, as far as the polar zones. Nerve-fibres form a simple plexus around the intrafusal fibre. 

**On the 5th day after birth** (Plate 1, fig. A) a lymphatic space about 10 \( \mu \) wide begins to develop between the capsule and the equatorial zone of the spindle. Most spindles are as much as 500 \( \mu \) long and about 30 \( \mu \) wide in the equatorial zone. The nuclei in the equatorial zone gradually become less basophil, lighter, and poor in chromatin. In the myotubal zone the nuclei, rounded at the ends, are placed one behind the other in the centre of the muscle-fibre and surrounded by protoplasm containing striated myofibrils. In the distal polar zone the nuclei are placed at the periphery and the muscle-fibres differ from extrafusal fibres only by being of smaller diameter. The capsule of connective tissue is already differentiated in the polar zone of the spindle. Between the 5th–10th day the muscle-spindle acquires its motor innervation. The motor-fibres form primitive end-plates in the polar zone, while the proprioceptive fibres run freely along the intrafusal fibres and produce a plexus with wide loops. 

**Till the 10th day** the diameter of the intrafusal fibre increases mainly in the equatorial zone. The periaxial lymphatic space also widens, so that the spindle reaches 40 \( \mu \) in width at the equator (Plate 1, fig. B). 

**By the 15th day** after birth the lymphatic space is already formed in the distal polar zone, but this remains of capillary dimensions. There is no basic change at the equatorial zone, which is generally about 40 \( \mu \) on cross-section and about 80 \( \mu \) long. Proprioceptive fibres form loose plexi and also accompany the intrafusal fibres, with a wave-like course. It is not yet possible to differentiate between annulospiral and flower-spray endings. 

**By the 25th day** after birth longitudinal growth of the spindles has taken place, so that they have become almost 1 mm. long, though neither the length of the equatorial zone nor the width of the intrafusal fibres has changed significantly. The equatorial zone at this time is 60 \( \mu \) long, the myotubal zones are 60–100 \( \mu \) long, the rest being the polar zones. The lymphatic space widens, measuring 16 \( \mu \) at the most, so that the total spindle diameter increases to 50–60 \( \mu \). The annulospiral and flower-spray endings are being formed (Plate 1, fig. C), and the spindle has all the morphological characteristics of a mature neuromuscular receptor.
B. The morphogenesis of intrafusal fibres in the denervated calf-muscle

1. Section of the sciatic nerve performed in rat foetuses on the 19th–20th day of intra-uterine development

At this time the differentiation of intrafusal fibres has begun by the division of nuclei in the myotubes in the neighbourhood of the branching of proprioceptive nerve-fibres.

Three days after the denervation (4 animals) spindles containing 1–2 intrafusal fibres in the equatorial zone, which may already be 60 μ long, are to be found in new-born rats in sections of the calf-muscles normally innervated. Similar structures do not occur in the denervated extremity. Occasionally muscle-fibres of myotubal character were observed, containing either long nuclei with a fine chromatin structure, or fibres with elongated nuclei arranged closely end-to-end (Plate 2, fig. F), often locally basophil to different degrees. Some of these nuclei are pycnotic with a tendency to karyorrhexis. Occasionally oedematous fibres occur in the musculature. Relatively often very fine fibres, mostly without myofibrillar structure, with elongated nuclei filling up the whole cross-section and placed at considerable distance from each other, may be found.

Very occasionally karyorrhexis was observed in oedematous muscle-fibres. In some of the muscle-fibres an uninterrupted chain of 5–6 tubular nuclei is present either darkly or lightly stained (Plate 2, fig. G). In the lighter nuclei the nuclear membrane at some places disappears and a strand of feebly stained karyoplasm remains in the muscle-fibre and is continued by basophil nuclei about 2 μ wide, surrounded by sarcoplasm, at both ends. All these structures occur quite exceptionally.

Four days after intra-uterine neurotomy (3 animals) thin basophil nuclear chains may be observed in exceptional cases, with overlapping, badly defined boundaries. These nuclei are not always surrounded by sarcoplasm. At both poles, however, there are remnants of sarcoplasm in the form of eosinophil threadlike endings. In one case a similar structure with disintegrating pycnotic nuclei was observed and these were joined together by a fine basophil bridge, which was evidently a remainder of the nuclei of the central zone.

Five days after intra-uterine neurotomy (3 animals) muscle-fibres containing elongated nuclei placed medially end-to-end are rarely to be found in the denervated musculature. In some of these the sarcoplasm is almost non-existent. The structures described occur very rarely—one in 4 or 5 longitudinal sections—while on the control side it is possible to count 25–30 muscle-spindles in the same number of sections.

Seven to ten days after intra-uterine neurotomy (5 animals) simple atrophy takes place in the denervated musculature and disintegration of individual muscle-fibres is rarely to be seen. In sharp contrast stands the musculature of the normal limb, where muscle-spindles are as long as 200 μ.
2. Section of n. ischiadicus in rabbit foeti 7 days before birth

The musculature at this time (i.e. the 21st day of intra-uterine development) is at the myotubal stage. The nuclei are placed centrally and the myofibrils at the circumference of the myotube. In some of these myotubes there are 5–6 nuclei arranged end-to-end. This nuclear chain, which is considered to be the initial stage of differentiation of the intrafusal fibre, occurs sometimes parallel in two neighbouring myotubes (see Plate 2, fig. H).

In new-born rabbits the neuromuscular spindles in normal muscles are in an advanced stage of development (see Plate 2, fig. I). The equatorial zone, containing cylindrical nuclei which fill up the whole lumen of the fibre, is 100 \mu long, and the myotubal and polar zones can easily be recognized. The connective tissue capsule and the periaxial lymphatic space are formed throughout the whole length of the spindle.

In the denervated musculature of two rabbits, which had survived the operation, on the other hand, it is not possible to find the least trace of the differentiation of spindles after birth, i.e. 7 days after denervation. The muscles are atrophic with small muscle-fibre diameters and without connective tissue proliferation (see Plate 2, fig. J). There are no structures nor end-to-end arrangements of nuclei in these muscles recalling the intrafusal fibres.

3. Section of n. ischiadicus in new-born rats

When the operation is performed in the new-born rat, the nuclear bag of the muscle-spindle is already differentiated.

Five days after neurotomy (4 animals) this differentiated equatorial segment is present, and only exceptionally can a reduction of the number of nuclei in some of the intrafusal fibres or symptoms of vacuolar degeneration be observed.

Ten days after neurotomy (3 animals) intrafusal fibres in the denervated muscles can hardly be discerned. The characteristic nuclear bag disappears completely and the intrafusal fibres disintegrate. In many cases an abundant invasion of phagocytic elements may be observed inside the spindle, which is formed by a connective tissue capsule closely adhering to the fragmenting intrafusal fibres.

4. Section of n. ischiadicus in 20-day-old rats

In the 20-day-old rats at the time of neurotomy the non-nervous component of the spindle is already morphologically mature.

Five days after the operation (4 animals) no changes were observed in the muscle-spindles of the denervated musculature.

Ten days after denervation (2 animals) slight atrophy of the muscle-fibres has occurred and a hardly noticeable shrinking of the nuclei in the equatorial zone.

DISCUSSION

It has been demonstrated that further differentiation of neuromuscular spindles is stopped by denervation performed in the initial stages of develop-
ment. In rare cases further division and arrangement of nuclei end-to-end in chains may take place in denervated myotubes, but the typical nuclear bag of the intrafusal fibre was never seen.

In new-born rats, in the musculature of which the nuclear bag of the intrafusal fibre is already almost differentiated, the intrafusal fibres atrophy in 10 days time and disintegrate. Innervation is evidently necessary for the normal course of development of the muscle-spindle.

In 20-day-old rats, on the other hand, the spindles of which are morphologically mature, it is possible to observe a hardly noticeable atrophy of the nuclear bag after 10 days. The loss of innervation leads then, at an early stage of development of neuromuscular spindles, to the arrest of the differentiation of intrafusal fibres. In new-born rats rapid disintegration of neuromuscular spindles occurs and in young rats gradual atrophy takes place as in adult animals.

In peripheral structures different degrees of trophic and morphogenetic dependence on innervation can be found. Non-nervous components of encapsulated skin receptors in mammals disintegrate after longer periods of denervation and their differentiation is again realized by the morphogenetic influence of regenerating nerve-fibres on the surrounding connective tissue in an even greater number than normal (Holobut & Jalowy, 1953; Dijkstra, 1933). According to these reports and our own experimental results, it seems probable that the trophic and morphogenetic dependence of receptors on their innervation may be greater than that of other peripheral structures.

The muscle-spindle as a receptor differs considerably from other encapsulated sensory endings in its structure as well as its innervation. The main difference lies in the fact that muscle-spindles have a dual innervation: proprioceptive in the equatorial zone and motor in the polar zones. By severing n. ischiadicus both the afferent and efferent innervation is affected. Is it possible under these experimental conditions to ascertain which of these two sets of nerve-fibres induces the differentiation of the spindles? This question may be answered by taking into account the normal development of muscle-spindle innervation. Motor innervation of intrafusal fibres is not completed until after birth, as has been demonstrated by impregnated sections and by the histochemical reaction to specific cholinesterase, which does not appear in the polar segments of muscle-spindles before the 5th day after birth (Zelená, 1957). Before birth the developing intrafusal fibres are only innervated by proprioceptive fibres with characteristic branching. The differentiation of muscle-spindles is stopped after intra-uterine denervation in consequence of the loss of proprioceptive nerve-fibres.

Is it possible that the retardation of muscle-spindle development is caused by loss of function, i.e. the specific function of the muscle, which cannot be realized after denervation? Our experiments do not answer this question directly, because section of n. ischiadicus removes motor as well as proprioceptive innervation of the muscle, so that only passive movements of the denervated extremity may be performed.
It has been shown, however, in denervation experiments on muscle-spindles of adult animals (Tower, 1932) that the elimination of proprioceptive nerve-fibres by extirpation of spinal ganglia leads to atrophy of the equatorial zone of the intrafusal fibres with a decrease in the number of nuclei even though motor innervation is intact. This atrophy therefore takes place, although specific function of the polar zone is preserved and the equatorial segment is tensed and relaxed as in normal spindles. If specific function does not prevent the atrophy of the equatorial segment in adult animals, it is possible to assume that neither during ontogenesis do nerve-fibres influence the differentiation of myotubes by way of the specific function, i.e. contraction of intrafusal fibres, since under normal conditions the polar zones of the spindle are not innervated until the differentiation of the equatorial segment had been roughly completed.

How then is the morphogenetic influence of the nervous system on the peripheral structures realized? The trophic influence of the nervous system is doubtless of a biochemical nature and is realized, most probably, as an effect of the nerve-endings on the metabolism of the innervated structure. The analysis of this influence is a difficult biochemical and histochemical problem. Analysis of the trophic influence of the motor neuron on skeletal muscle (Vodička, 1956) contributes, amongst others, towards a solution of the basic problem, the definition of the biochemical nature of the trophic influence of the nervous system.

It is interesting to note the different response of intrafusal fibres to denervation during differentiation and of intrafusal fibres in spindles morphologically mature. If the trophic influence of the nervous system is considered primarily to be in the activation of anabolic processes in tissue metabolism (Gutmann et al., 1956), it is probable that during development and structural differentiation, where anabolic processes far surpass catabolic processes, the developing tissue is more sensitive to denervation because the activation of anabolic processes by the nervous system is eliminated. Adult structure is in comparison more in equilibrium as far as anabolic and catabolic processes are concerned, maintenance of the metabolic equilibrium is more stabilized, and denervation is followed by atrophy, i.e. by a gradual predominance of catabolic over anabolic processes.

**SUMMARY**

1. The development of muscle-spindles in the calf-muscles of the rat was followed under normal conditions and after section of n. ischiadicus.

2. The first differentiation of muscle-spindles in the muscles of the hind limb of the rat was observed on the 19th day of intra-uterine development and their morphogenesis is complete by the 25th day after birth.

3. After section of n. ischiadicus in rat foetuses on the 19th–20th day of intra-uterine development, differentiation of muscle-spindles does not take place; neither intrafusal fibres nor the connective tissue capsule are formed. Since only proprioceptive fibres have innervated the normal muscle at this time, it is the
loss of the morphogenetic influence of these nerve-fibres, that prevents differentiation. Similar results were obtained in rabbits denervated 7 days before birth.

4. When section of n. ischiadicus is performed in new-born rats, disintegration of intrafusal fibres in which the equatorial segment was already differentiated at the time of denervation occurs within 10 days.

5. Denervation in a 20-day-old rat, the muscle-spindles of which are already morphologically mature, leads after the same interval, to a slight atrophy in the equatorial zone of the intrafusal fibres.

6. The mechanism of the morphogenetic influence of the nervous system and its changes during ontogenesis are discussed.

ACKNOWLEDGEMENTS

I should like to thank Dr. E. Gutmann for his valuable advice and encouragement during this work, Mrs. E. Mařáková and Mrs. M. Sobotková for technical assistance, and Mr. Kubec for the photographs.

REFERENCES


EXPLANATION OF PLATES

PLATE 1

Fig. A. Longitudinal section through the calf musculature of a rat 5 days old. a, spindle equatorial zone. Azan. × 200.

Fig. B. Diagonal section through the neuromuscular spindle of a 10-day-old rat. a, equatorial zone; m, myoblastic zone; c, capsule. Haematoxylin-eosin. × 200.

Fig. C. Longitudinal section of neuromuscular spindle in a 25-day-old rat. a, equatorial zone; c, capsule; I, primary endings at the equator; II, secondary endings in the myoblastic zone. Gross-Bielschowski. × 200.

PLATE 2

Fig. D. Myotubes in the calf musculature of a rat foetus 3 days before birth. Van Gieson. ×1,100.

Fig. E. The equatorial zone of an intrafusal fibre in a new-born rat. c, connective tissue capsule. Haematoxylin-eosin. × 1,100.

Fig. F. Nuclei closely ranged end-to-end in myotubes of denervated musculature in a new-born rat 3 days after section of the sciatic nerve. Haematoxylin-eosin. × 1,100.

Fig. G. Chains of deformed nuclei in a swollen myotube of denervated musculature in a new-born rat 3 days after section of n. ischiadicus. Haematoxylin-eosin. × 1,100.

Fig. H. Myotubes in a rabbit foetus 7 days before birth. a, chains of nuclei in myotubes. Haematoxylin-eosin. × 400.

Fig. I. The central segment of a spindle in a new-born rabbit. a, equatorial zone; m, myotubal zone; c, capsule. Haematoxylin-eosin. × 400.

Fig. J. The atrophy of denervated musculature in a new-born rabbit 7 days after section of the sciatic nerve. Haematoxylin-eosin. × 200.

(Manuscript received 23 : x : 56)
J. ZELENÁ

Plate 1