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CHANGES IN MOTOR NERVE ENDINGS IN FAST- AND SLOW-TWITCH MUSCLES OF NORMAL AND ENDURANCE-EXERCISED RATS

By

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ABSTRACT

CHANGES IN MOTOR NERVE ENDINGS IN FAST- AND SLOW-TWITCH MUSCLES OF NORMAL AND ENDURANCE-EXERCISED RATS

By

Kenneth Ellis Stephens

Morphological change in neuromuscular axonal terminals was examined using a combined silver-cholinesterase stain in 100 male Sprague-Dawley rats. Predominantly slow-twitch (adductor longus and soleus) and fast-twitch (gastrocnemius and rectus femoris) muscles were taken from sedentary animals at 6-28 weeks of age as well as from rats subjected to 4-16 weeks of intense endurance exercise. The exercise program initiated at 12 weeks of age was progressive in nature with the final group of rats running at 36 m/min for a total of 2 hours per day, 5 days per week. Motor nerve endings were categorized into defined morphological classes. In maturing animals the number of accessory endings increased significantly in slow-twitch muscles from 6 to 16 weeks, at which time it plateaued in the soleus and slightly decreased in the adductor longus. In fast-twitch muscles the increase ceased at 12 weeks of age, with a subsequent leveling off after that. Double endings also increased in the predominantly slow muscle up to between 20 and 24 weeks. Conversely, fast-twitch muscle displayed no such increase and, indeed, displayed less than 1% double endings after puberty. The number of branched endings appeared to decrease post-puberty in all muscles to below 3% where it remained for the duration of the study. Simple endings decreased significantly between 6 and 20 weeks in slow-twitch while in fast-twitch muscles the decrease was seen between 6 and 12 weeks of age. Examination of exercise data indicated few changes were brought about by functionally overloading the muscles. This study suggests that 1) significant differences exist in the neuromuscular axonal terminals of post-pubertal fast-twitch and slow-twitch muscles; 2) with post-pubertal maturation an increase in the complexity of these terminal patterns develops in slow-twitch muscle which is not present in fast-twitch muscle; 3) exhaustive endurance exercise appears to have little effect on the modification of motor nerve endings. This indicates that normal maturational nerve ending changes are not due to alterations in neuromuscular activity.

DEDICATION

To Ellis, Margaret, and my Family--for their continuous encouragement and support.

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CHAPTER I

THE PROBLEM

Using various histochemical techniques in conjunction with physiological, biochemical, and anatomical methods, three basic fiber types have been categorized in normal mammalian muscle (20,49,95). These fiber types are dynamic and capable of responding morphologically as well as metabolically to a wide variety of stimuli such as denervation (15,51, 61,63,75,77,98,119), cross-innervation (15,17,18,27,63,119), inactivity (9,12,119), exercise (25,50,69,70), or ageing (22,26,51,58,60,85,119).

The neuromuscular junction has been shown to bear specific characteristics relative to muscle fiber type. Under light microscopic examination varying degrees of ending/endplate complexity have been identified (6,30,31,43,56,66,79,90,94,115), and in some cases categories of terminal arborizations can be associated with specific fiber types (79,117,120). Examination of the post-synaptic portion of the endplate with the electron microscope has revealed distinct adaptations in terms of number and structure of junctional folds (23,40,42,87,91,92,93,97, 109), endplate mitochondria (40,42,44,47,87,91,92,93), vesicle number or shape (44,47,52,87,92,97,109) or sole-plate sarcoplasmic development (87,91,92,93,97). Likewise, the morphology of the presynaptic portion of the junction (i.e., the terminal axon/ending) is closely linked to the muscle fiber type it innervates. Characteristics have been identified here in terms of terminal boutons (79,94,116), mitochondria (44,93), axoplasmic vesicles (44,52,93,109), collateral sprouting (6,43,116), or intra-endplate sprouting (94).

Observations in normal animals have indicated that, like muscle, fiber types terminal endings/endplates are not static (33,49,71,84,94, 116,117). In the mammalian neonate, polyneuronal innervation (multiple axonal inputs into a single endplate) is characteristic (73,99,117,119). With maturation, polyneuronal innervation is lost by withdrawal of redundant terminal axons--leaving an adult pattern of unineuronal innervation (73,99,117,119,125). It has been shown, however, that this adult pattern is subject to mutation during the normal ageing process either through ending/endplate degeneration and regeneration (33,71) or via continuous elaboration of terminal arborizations (116,117). Further, the neuromuscular junction has been shown to be morphologically responsive to treatments such as denervation (34,63,119,125), reinnervation (34,63,84, 119,125), cross-innervation (33,37,84,119,125) and change in activity level (9,119,125).

Statement of the Problem

The overall objective of this investigation was to determine the effects of both maturation and an induced exhaustive endurance exercise program on the morphology of the motor endplate and its respective terminal axon in selected slow-twitch and fast-twitch muscles of male albino rats.

The investigation sought to provide insight into the following questions:

1. What are the morphological differences in motor nerve endings between slow-twitch and fast-twitch muscles?
2. How does the motor ending structure, and consequently the innervation pattern, respond to chronic physical activity (endurance exercise) in these same muscles?
3. Are there changes in the motor ending structure in fast- and slow-twitch muscle which could be associated with maturation?

Significance

Information concerning synaptic plasticity under varied circumstances is provided by the present study. Quantitative observations of various types of motor nerve endings obtained at selected points during maturation indicate the normal adaptive changes which occur and will be useful in differentiating these from pathological conditions of myoneural junctions. In addition, any such changes noted which are fiber-type specific should prove useful in the further understanding of normally and abnormally occurring nerve ending alterations. Finally, the specific quantitative evaluation of these endings in normal and exercised animals gives insight into adaptations in the terminal ramification brought about by increased workload.

Limitations of the Study

1. The results of this study are restricted to normal male, Sprague-Dawley albino rats, and absolute values may prove to be species and/or strain specific.

2. The results are similarly limited to the muscles cited (soleus, adductor longus, gastrocnemius, and rectus femoris) and their respective fiber type populations.

3. The study was further restricted by limitations in histochemical techniques which preclude at present, the evaluation of quantitative enzyme concentrations in individual muscle fibers and the subsequent association of these fibers with specific motor ending structures.

4. While quantitative evaluation of the training program used was limited due to undetected deficiencies in the controlled-running wheels and the master control unit, it was established qualitatively that the exercise program was a very demanding endurance experience for the animals.

5. As four general sectioning and staining sessions were required to process all tissue, intersession variability may have influenced some of the results.

CHAPTER II

REVIEW OF RELATED LITERATURE

Introduction

The purpose of this investigation was to examine the morphological plasticity demonstrated at the myoneural junction of maturing and endurance-exercised rats. Terminal axons and their respective motor endings were demonstrated in selected fast- and slow-twitch hindlimb muscles and were subsequently categorized in accordance with a pre-defined criteria (117).

The following review of literature related this examination with current research papers or theories providing insight into the differences in motor ending morphology in specific muscle fiber types associated with ageing or in conjunction with induced exercise. An attempt also has been made to include review of that literature which might explain the mechanisms for or functional significance of observed differences.

Initiation of Nerve-Muscle Connections

The initiation of nerve-muscle connection is characterized at the onset by the appearance in the myotomes of "exploratory fibers" or "pathfinder nerves" (64). While relatively few in number, these fine nerve outgrowths are thought to provide, for subsequent developing axons, a guidance mechanism or path along which they can course until a peripheral target organ is reached (64). Axonal growth, both primary and secondary, is readily evident among myoblasts in the form of growth cones (64.72).

It is at this point, while the axon terminals are still quite separate from the myoblasts that primitive neuromuscular junction traits appear. Within the myoblasts, non-localized acetylcholine (ACh) receptors are being synthesized (45) and acetylcholinesterase (AChE) is present (110). Choline acetyltransferase (ChAc) has been detected at this time in the muscle mass (119). As ChAc has been proven to be localized to nerve terminals (65,119), this indicates the ability of the nerve terminal to synthesize ACh prior to formal contact with the muscle cell.

The formal relationship between nerve and muscle thus first occurs between developing nerve terminals capable of releasing the neurotransmitter ACh and myotubes which have differentiated to the point at which they are capable of response to the nerve stimulus (i.e., they have increased membrane chemosensitivity to ACh) (266).

The interdependence of nerve and muscle is established here for further existence or development. Motor axons failing to make permanent peripheral contacts are redundant and die back (72,119) while those which succeed in connecting with peripheral organs apparently mature further (73,119). Myotubes which remain functionally uninnervated cease to differentiate further, atrophy, and are replaced with fat

(24,38,72,119). In this manner and due to the outgrowth of a large excess of motor axons full neurotization is normally ensured in the developing embryo.

Primitive Neuromuscular Junction

The earliest morphological demonstration of myoneural junctions in rats occurs at approximately 16 days *in utero* (8,38,76,113,114), which coincides with the first signs of muscle contraction which can be elicited from rats by electrical stimulation (8,113). The appearance of visible myoneural junctions is obviously species specific as Hirano (68) and Bennett and Pettigrew (8) reported their presence between seven and nine days *in ovo* in chick embryos, whereas Fidzianska (46) reported nerve-muscle contacts in human fetal muscle between nine and 16 weeks.

Terävainen's ultrastructural analysis (114) of the morphogenesis of the rat myoneural junction provided a basis for our current understanding of the developmental stages. In it he suggests that initially, presynaptic axons with ACh-containing vesicles approach the developing myofibers, and that these terminals induce changes in the postsynaptic membrane which result in its thickening. At this stage the primary synaptic cleft is wide and apposition is irregular. With the narrowing of the cleft to roughly 500Å, the first visible, primitive junction was noted to be characterized by "teloglial-covered axon terminals apposed to the thickened but unfolded plasma membrane of the muscle fiber." The presence at this junction of 1-8 axon terminals, side by side, containing terminal vesicles, mitochondria and occasionally agranular reticulum was reported. The formation of secondary synaptic clefts was seen to occur in the postsynaptic membrane between 20 days pre- and 4 days post-natal. These relatively immature, finger-like invaginations were found to develop to adult status (increased in number and depth) between five and ten days post-natal. Finally Teräväinen noted increases in numbers of mitochondria and vesicles in axon terminals with age, as well as separation of terminals at the junction between the 10th and 16th post-natal day. Sequentially, these findings were in relative agreement with those of Kelly and Zacks (76) for the rat, Hirano for birds (68) and Bennett and Pettigrew (8) for both.

Post-natal examination of the maturation of junctions was carried out using kittens of various ages by Nyström (88). Using the classical definitions for "terminaisons en grappe" and "terminaisons en plaque" developed by Tschierau in 1879 he attempted to differentiate in "slow-red" and "fast-white" muscles between neuromuscular junctions. At no time were "en grappe" or grape-like clusters of endings seen; however, despite the fact that both soleus and gastrocnemius muscles displayed "en plaque" (plate-like) endings, there were distinct differences both in structure and development. During the first week the only difference noted was in size. The soleus, which had larger diameter muscle fibers, also had larger endings. This relationship between endplate size and muscle fiber diameter persisted into adulthood. At approximately two weeks of age differentiation of endplates from primitive disc-like structures was initiated in gastrocnemius from the center of the ending and in the soleus junctions from the periphery. By two months, the endplate of the "fast-white" muscle had developed distinct morphological characteristics which enabled its differentiation from the soleus terminal arborization: the former was well established and compact while the later appeared somewhat "fluted and wrinkled". In adult animals the gastrocnemius displayed endings which were fairly wide spreading, long and smooth. This was in contrast to the shorter, more compact, wrinkled soleus terminal. Investigation of other "fast-white" muscles revealed endplates with traits similar to those found in gastrocnemius muscle. Other "slow-red" muscles showed the characteristic ending displayed by soleus muscle. Nyström failed to note any true mutli-innervation of endings at any stage of development.

Polyneuronal Innervation

Terävainen's observations (114) of 1-8 axons at endplate areas, and their subsequent separation from each other during development combined with the differences observed by Nyström (88) in endplate morphology and development in different fiber types is indicative of the plasticity of the myoneural junction during maturation. In the last decade much of the focus on plasticity in the neuromuscular junction has centered on polyneuronal innervation and its elimination.

In 1970, Redfern (99) examined endplate potentials (e.p.p.s) in diaphragm muscles of neonatal rats during the first four weeks of life. By isolating intact the diaphragm and phrenic nerve in curarized mammalian Ringer's solution and subsequently stimulating systematically through the phrenic nerve at various strengths while recording e.p.p's intracellularly at the middle of the muscle fiber, Redfern was able to observe the addition or subtraction of "units" to the e.p.p. and the resultant complex e.p.p. This he accomplished by examining recordings of step-wise increases in amplitude of e.p.p's with progressive stimulus strength increases. These step-wise increases were taken to indicate recruitment of axon terminals of varied thresholds. In several cases however, he surmised addition of units where abrupt jumps in e.p.p. amplitude were present. In this manner he concluded that there were "functional connections between more than one axon in the nerve trunk and a single neonatal muscle fiber and that these axons have different latent periods and thresholds to nerve stimulation." During the first week of existence, there were e.p.p's present with such multiple units, usually 2-4. Redfern noted a gradual shift to more single unit e.p.p's during the second week and indicated that by 16-18 days of age multiple units were rare. This decrease was thought, by him, to be representative of loss of polyneuronal innervation through elimination of superfluous nerve branches.

Using kittens three days, two weeks, and six weeks of age, Bagust _et al_. (4) sought to establish whether Redfern's observations were attributable to true _polyneuronal_ innervation or were, in fact, a reflection of _multiple_ innervation of one endplate by a single axon. Isometric contractions were elicited from the soleus and flexor hallicus longus muscles by stimulation of their respective ventral roots. The tetanic tension recorded by _in toto_ nerve stimulation was significantly less than that attained by stimulating split (equal) nerve preparations in either muscle at either three days or two weeks. This was taken to be indicative of dual innervation of some fibers by more than one axon;

i.e., polyneuronal innervation. The differences were initially large at three days, less at two weeks, and small in the flexor hallicus longus while absent in the soleus at six weeks. Apparently polyneuronal innervation was removed by some mechanism during the first six post-natal weeks in the kitten, with the developing fast-twitch muscle lagging somewhat behind in its elimination. The authors suggested that through polyneuronal innervation the adult pattern of innervation could be more precisely linked to the demands of the nervous system through loss of less useful connections.

In a subsequent report (122) the authors confirmed the preceding findings and indicated that reduction of polyneuronal innervation was not attributable to increased muscle fiber numbers. Total fiber counts at all ages considered were not significantly different in either muscle. Preliminary attempts at histological examination suggested the presence of multiple innervation of myofibers at two weeks but were unable to distinguish <u>multi</u>- from <u>polyneuronal</u> innervation. Axon counts, preliminary also, indicated increases of approximately 15% between two and six weeks in both muscles which corresponded to those observed by Nyström (88). The increase in axon counts was thought in part, to be responsible for decreased motor unit sizes in maturing animals. It may also be considered reflective of the extreme plasticity of the innervation pattern during this stage of development.

In rat diaphragm examinations, Bennett and Pettigrew (8) confirmed the initial developmental sequence for innervation pattern using a combination of histochemical, ultrastructural, and electrophysiological techniques. They established the presence of at least three synapses at a single site on developing myotubes by examining rise times for m.e.p.p's and also observing only one localized spot of ChE per myotube in association with several silver-stained axons. As with previous investigations, the number of nerve-muscle contacts was reduced to a unineuronal adult pattern between the second and fourth weeks. Bennett and Pettigrew also suggested a correlation between quantal content of the e.p.p. and the size of the nerve terminal, suggesting that with maturation more quanta are available for release on stimulation. Finally it was stated that the pattern of innervation in focally innervated muscle was established by the initial axon making contact with the myotube. While this initial contact was at random, subsequent contacts were made only at the initial endplate site in normal animals. It appeared to the authors that the first axon induced a change in surrounding myotube membrane which rendered it refractory to further innervation.

This same report (8) provided insight into the species-specific nature of the nerve-muscle contact. A description of the sequence and development of chick anterior latissimus dorsi muscle indicated a distributed-innervation pattern in which endplates were established along the length of the myotubes at distances greater than 170 μm and at relatively regular intervals. These sites also were supposed to have been innervated by a single axon initially, followed by transitory multiple innervation, and bear an "en grappe" configuration for ChE deposits. Electrophysiological examination showed changes in stimulus strength resulted in alteration of latency and rise times as well as amplitude of e.p.p's. The endplates apparently received a multiple and distributed innervation detectable at nine days incubation. Multiple innervation was generally removed by four weeks post hatching and the membrane areas between endplates appeared refractory to further axonal termination.

In an effort to determine if membrane refractoriness is determined by nerve type, Bennett and Pettigrew (7) performed a series of experiments during development on the focally innervated rat tibialis anterior and on the avian posterior (focal innervation) and anterior (distributed innervation) latissimus dorsi muscles. Reinnervation of tibialis anterior muscle indicated original synapse sites as well as ectopic snyapses located in areas of new muscle growth (lengthened through addition of sarcomeres) were innervated during development. However, in muscles denervated at progressively longer times from birth, the percentage of these ectopic endplates was decreased. Consequently it was concluded that the muscle membrane present at denervation was still refractory to additional synapse formation for some time. The original synaptic site seemed the preferred junction as following the sixth postnatal week, almost all ectopic synapses were gone. In all cases "en plaque" endplates were characteristic of the area and extensive collateral sprouting was noted during reinnervation. Anterior latissimus dorsi muscles, normally of the "en grappe" type, also showed reinnervation at original sites as well as at ectopic locations on muscle added during the denervation period. As in the original work, several hundred micrometers separated endplates. Cross-innervation between anterior and posterior latissimus dorsi muscles produced myofibers with synapses which were characteristic of their new nerve. Thus the anterior latissimus dorsi became focally innervated with "en plaque" terminals, and electrical properties characteristic of fast nerve-muscle synapses. The posterior latissimus dorsi received "en grappe" terminals and a distributed-innervation pattern.

These studies (7,8) indicated that the "nerve determines the pattern of synapses over an effector during development, and therefore the pattern of preferred sites of synapse formation in reinnervated and cross-innervated adult muscle." The mechanism by which the nerve establishes this pattern appears to be related to its ability to render the muscle membrane refractory during development.

Reports by Brown _et al_. (13,14) confirm results from previous investigations (4,7,8,99,114,122). They recognized genuine functional polyneuronal innervation in neonatal soleus muscles of rats at single synaptic sites using both electrophysiological techniques and histochemical ChE staining methods. Elimination of polyneuronal innervation was most apparent between the tenth and thirteenth day when fibers exhibiting multiple inputs decreased from 91% to 2.5%. As no decrease in total number of motor units was noted during this period electrophysiologically, the reduction in number of terminals per site was attributed to a decrease in motor unit size. Furthermore, as there was a wide spread of these sizes which was considerably greater in young than adult animals, the authors concluded that rather than a synchronous loss by each motoneuron of a fixed percentage of its peripheral terminals, the elimination process acted predominantly on the terminals of the largest motor units.

In their latter report (14) the effects of partial denervation, denervation and cross-innervation during development were examined. Partial denervation of the soleus muscle on one side indicated considerable slowing in the elimination of multiple terminals from those muscle fibers still associated with nerve supply. There was still evidence of polyneuronal innervation two weeks after the control side had developed its mature status. The presence of atrophied and hypertrophied fibers, and the apparent lack of axonal sprouting, as is visible in adult muscles (41,54,118), led to the conclusion that withdrawal of synapses can persist even while some myofibers become denervated. The existence of an upper size limit (i.e., a maximum number of fibers innervated) for developing motor units was advocated. Denervation during the first two days post-natal by nerve crush at the nerve entry point into the muscle resulted in reinnervation during the first two weeks in the general vicinity of the endplate band at both original and ectopic sites. Hyperinnervation was common and its removal proceeded at a similar rate to that in normal muscle, indicating neither age of terminals nor early muscle inactivity affected elimination of additional terminals. Cross-innervation experiments indicated no synapses could be formed by a foreign nerve in developing muscle unless the original nerve to the soleus was cut or crushed. In this event, foreign synapses were observed in both a distributed pattern about 1 mm apart (minority) and focally. In both cases, polyneuronal innervation was removed approximately one week later than normal (i.e., during the third week), although in some cases dual synaptic sites were maintained (always more than 1 mm apart) indefinitely. In summarizing their results, Brown _et al_. (13)

indicated that survival of a specific terminal or a muscle fiber was highly dependent on its motor unit size, with those emanating from smaller units having an advantage. This competitive interaction among synapses was also thought to involve muscle-nerve communication of an unspecified nature.

Bixby and VanEssen (10) using a variety of muscles in the rabbit again illustrated that elimination of polyneuronal innervation was not attributable to increases in myofiber numbers. Neither was there a loss of myelinated axons from the soleus nerve (237 at day 2 versus 233 at day 16). Elimination was attributed to retraction of synapses by individual motor nerves. No differences were detected in either rate or onset of elimination in muscles of varied contractile properties (soleus and extensor digitorum longus). Differences in onset of up to one week were noted when body position of the muscle was considered. Those located rostrally had earlier onset times.

Using histochemical methods, Riley (103,104,105) corroborated the findings of the largely physiological studies preceding. In kitten soleus muscle he concluded that myofibers generally were innervated at a single endplate by 2 or more terminals in 72% of the cases examined in newborns. This percentage decreased to approximately 3% by 4 weeks and 1% by six weeks. The method of analysis employed indicated the terminals involved were from separate neurons, and consequently multiple terminals per endplate were deemed as anatomical substrate for polyneuronal innervation. Findings in the soleus muscle of the rat paralleled the cat findings, although on a somewhat different time course. Seventy-three percent of the endplates in 11-day-old rats showed two or more terminals. By the end of the second week, only 9% were polyneuronally innervated. "Retraction bulbs", initially appearing as a swelling near the edge of the endplate and at the end of a fine axon, were seen. Advanced stages of these bulbs generally showed them as an oval swelling at the end of an axon and adjacent to the innervating axon. Riley suggested the final retraction stage to be complete resorption by the parent axon. As retraction bulbs were visible at various distances from the endplate at 11-15 days, during the period of greatest elimination, a pattern of non-synchronous retraction of terminals was thought to reflect the mechanism for removal. Also observed however was evidence suggesting in situ degeneration of redundant terminals, although retraction seemed the preferred mode of removal.

Riley subsequently reported that the reduction in polyneuronal innervation was not due to an increase in fiber number, as these remained constant after birth in his investigation (105). Furthermore, values for the rat soleus muscles used showed roughly 50% of the fibers were Type II between 11 and 15 days, indicating that fibers of both types were multi-innervated. Differentiation of fiber types apparently did not correlate with loss of polyneuronal innervation. Riley did indicate that more Type II fibers were multi-innervated, based on the relationship between fiber size, ATPase staining characteristics and terminal arborization observations, and suggested activity might be an important factor in determining fiber types and innervation pattern.

Subsequent work by Tweedle and Stephens (117) categorized endings during development as well as observing their morphogenesis. In two fast-twitch and two slow-twitch hindlimb rat muscles polyneuronal innervation was seen to disappear leaving the unineuronal adult pattern between the second and third weeks. Retraction bulbs present during this period confirmed, in part, Riley's observations (104). Between the third and fourth weeks, a large number of branched endings indicated possible continued synaptic reorganization. After a relatively quiescent period between the third and fifth week unineuronally innervated endplates became more complex, especially in slow-twitch muscles. This increase in complexity was thought possibly to reflect increases in myofiber size or workload.

The mode of elimination of polyneuronal innervation was focused upon by four papers (11,80,101,106) using ultrastructural techniques. Reier and Hughes (101) thought that Wallerian-like degenerative changes, seen primarily between 7 and 14 days in the sciatic nerve (both myelinated and unmyelinated) fibers of post-natal mice and rats, could account for the spontaneous degeneration during peripheral nerve maturation. Rosenthal and Taraskevich (106) reporting a high incidence of degeneration in their terminals, generally agreed with the theory extolling Wallerian-like degeneration as the mechanism of removal. Korneliussen and Jansen (80) found no evidence for degenerating intramuscular axons or terminals in their study using rat soleus muscle. Reduction in the number of terminating axons at endplates was observed between the eighth and sixteenth days as was the presence of ridge-like extensions from muscle fibers between groups of terminals. Schwann cells both between terminating axons and, in some cases, investing terminations were visible also. Removal of redundant terminals was thought to be by retraction into the parent axon.

Bixby (11), using denervated rabbit diaphragm, characterized the stages of degeneration of terminals in developing muscle. After comparing terminals and endplates in denervated with those in normal muscle, Bixby concluded that "no degenerating terminals were seen" in normal muscle, nor were there any signs of degeneration debris in Schwann cells at the endplates. He concurred strongly with the retraction theory. In examining the relationship between myelination and synapse elimination he found myelination lagged behind: terminals could be removed prior to myelination. While apparently not related to removal of redundant endings, irregular protrusions of terminals above the surface of the muscle fiber were seen, as were non-innervated areas of post-synaptic membrane. Both were taken to indicate the dynamic state of the developing endplate.

Apart from denervation-reinnervation (7,11,13) or cross-innervation investigations (7) where the muscle and ending were dormant for various periods during development, the effects of increased or decreased activity on the genesis of the myoneural junction and the terminal axon (to the adult state) have been examined only slightly. No study using forced exercise or activity during this time period has been conducted. Stimulation of embryonic spinal cord produced a distributed pattern of innervation in normally, focally innervated chick posterior latissimus dorsi muscle (102) as well as the normal polyneuronal innervation pattern at each site. It was not known whether all sites and/or terminals were functional, however.

Increase in activity resulting from electrical stimulation of the right sciatic nerve in rats produced a more rapid elimination of polyneuronal innervation, detected electrophysiologically in both stimulated muscle (soleus) and its contralateral counterpart (89). The reflex - activated, polyneuronal innervation reduction did occur later however than that on the stimulated side.

Reducing neuromuscular activity with curare in the developing chick posterior and anterior latissimus dorsi muscles resulted in prolongation of polyneuronal innervation as well as apparently increasing the number of terminals in contact with synaptic sites (112). By tenotomizing 4-day-old rat soleus muscle the mechanical activity of the muscle was suppressed (9). This decrease in activity resulted in a delay in the evolution of the adult pattern of innervation as polyneuronal innervation persisted well into the third week in tenotomized muscle. From these investigations and the observation that polyneuronal elimination coincides with a general increase in animal activity (99), it appeared that neuromuscular activity had an effect on innervation pattern and junctions.

Elimination Mechanism

The mechanism by which the neuromuscular system develops from a polyneuronal neonatal form to a unineuronal adult pattern is poorly understood. Generally, with no large scale death of motor neurons during the period of synapse disappearance and no apparent increase in number of muscle fibers, it would appear that the reduction in multiple inputs to synaptic sites is due to a reduction in motor unit size. Further, it appears this reduction is carried out through simple retraction of redundant terminals rather than via degenerative processes.

Specifically the mechanism for determination of which terminal is to survive has eluded investigators. Jansen *et al*. (73) and Willshaw (124) have indicated that random loss of terminals is an unacceptable mode as this would inevitably produce transient denervation of some developing fibers. Sufficient numbers of such denervated fibers, which survive for several weeks, are not visible during this period, nor is there a large amount of nerve sprouting which could explain their lack. Jansen _et al_. (73) further eliminated the possibility for competitive advantage of certain motor units, competitive or random selection by the muscle fiber, or selection by Schwann cells as accounting solely for the final scheme of innervation. They suggested that terminal elimination was related to the motor neuron's control of the ultimate number of synapses it made and that survival of specific endings was related to factors other than their level of activity or their simple ability to initiate contraction.

Vrbova's group of investigators (89,119) has presented the only explicit proposal for elimination of polyneuronal innervation. Their theory is based on the actions of proteolytic enzymes released from the endplate in response to ACh. They propose that the multiple terminals at the endplates progressively secrete more ACh as the neonate becomes more active. This ACh causes lyosomal enzymes to be released from the endplate region, or other cells in the vicinity, as well as providing a substrate for hydrolysis which ultimately will contribute to the formation of the acidic environment necessary for enzyme action. These lysosomal enzymes then diffuse into the interstitial space and affect nerve terminal membranes or the connections of the terminals with the endplate. In order to survive Vrbova _et al_. suggest that a terminal must be replaced by its motor neuron and that these neurons apparently have a finite capacity to support endings. Consequently, some terminal connections are lost as necessary replacement materials are unavailable, while others are maintained. Those lost are retracted once contact with the muscle fiber is broken. This then allows the parent neuron more replacement materials for its other branches and consequently facilitates its support of terminals at other endplates. A "feedback system" is advocated to insure the integrity of the surviving terminal. Should the terminal be being degraded faster than it can be regenerated, a decrease in ACh secretion will result in a decrease in the release, diffusion, and action of the lysosomal enzymes. The net result will be an equilibrium established between endplate and terminal which effectively guarantees maintenance of the unineuronal innervation pattern seen in the adult.

Increases in activity as seen in the study by O'Brien et al. (89) would be reflected, according to the theory, in earlier removal of polyneuronal innervation while decreases in activity, as in the Benoit and Changeaux investigation (9), would result in delayed removal of supernumary endings. Such apparently was the case. Vrbova and her colleagues also suggest this "lysosomal theory" may be applicable during the initial phases of neuromuscular development. Finally they propose the ACh-produced release of lysosomal enzymes may play an important role in the dynamic state of mature nerve endings.

Mature Innervation Patterns

Numerous review articles (16,29,41,55,67,115,118,119,123,125) provide the historical basis for current research into the myonerual junction.

Much of this work carried on between 1840 and 1940 focused on mature endings and, while unique, contributes little to our current understanding of the plasticity and adaptability demonstrated at the neuromuscular junction or to the apparent muscle-specific morphology of the terminal. Consequently review here of these articles has been omitted. Likewise literature pertaining to generally accepted morphological characteristics of motor arborizations has been deleted. Comprehensive reviews of such traits have been compiled elsewhere (1,29,34,48,97,125,126).

Specificity at the Neuromuscular Junction

Histological/Histochemical Light-Microscopic Studies

Morphological Characteristics (see Table 2.1)--Cole's initial investigations (30,31,32) emphasized the very specific nature of the terminal ending. His rather extensive 1955 publication (32) focused on endings in muscles of similar functions taken from normal vertebrates from elasmobranchii to mammalia classes. Using a gold chloride technique to identify terminals on teased fibers he reported a steady progression in ending characteristics from the tight, compact primitive ring of fish through the "terminaison en ligne" of amphibia and reptiles up to the "terminaison en plaque" or "grappe" of mammals. In addition, Cole reported both accessory endings and several instances of double endings, i.e., two motor endings on one muscle fiber. (From observation of the accompanying photographs, it appeared other categories of endings as described by Tweedle and Stephens (117) were also present including branched and multiples.) Cole attributed the differences in endings from varied classes to an adaptation to the particular functional needs of

Table 2.1. Morphological Specificity of Terminal Endings/Endplates -- Light Microscopic Results

Investigator(s)	Reference	Animal Model	Muscles Used	Techniques	Generalized Findings
Cole 1955	32	Varied	Iliotibialis (or homologue) Paravertebrals	Gold Chloride Stain on Teased Fibers	1. Species variations present. 2. "Terminaisons en plaque" (loose and compact), "terminaisons en liqne", "terminaisons en grappe", double endings and accessory endings seen. 3. No structural differences between muscles. 4. Functional adaptation presumed.
Cole 1957	31	Rat (150-399 gm)	Tongue Foot Interossei Diaphragm Rectus Femoris Gastrocnemius Intercostals Paravertebrals	Gold Chloride Stain or Teased Fibers	1. Intraspecies variations present. 2. Morphological characteristics of endings permit grouping. a. Compact "terminaisons en plaque" - skilled-movement muscles. b. "Terminaisons en grappe" and "en plaque" - respiratory muscles. c. Loose "terminaisons en plaque" - postural/ appendage muscles. 3. Double, accessory, and multiple endings seen. 4. Functional adaptation supported.
Hess 1962	66		Extraocular		1. Fast (white) fibers had single, "en plaque" endings. 2. Slow (red) fibers had many, "en grappe" endings.
Ogata 1965	90	Mouse	Gastrocnemius Adductor Magnus	SDH-Ch Combined Stain on Frozen Sections	1. White fibers had a large, complicated interlacing endplates. 2. Red fibers had a small, simple compact endplates. 3. Intermediate fibers had endplates with medium size and moderate development. 4. All endplates were "en plaque". 5. No neuronal input (axons) was demonstrated.

Table 2.1.— Continued

Investigator(s)	Reference	Animal Model	Muscles Used	Techniques	Generalized Findings
Korneliussen and Waerhaug 1973	79	Rat (200 gm)	Diaphragm	Methylene Blue on Teased Fibers	1. Morphological categorization of motor terminals. a. Type A - diffuse appearance; acutely branching and rebranching endings; minute rounded boutons. b. Type B - intermediate characteristics. c. Type C - compact dense appearance; intra-endplate branches thick and short; terminal boutons large and irregular. 2. Frequency of occurrence of terminal types corresponds with that of diaphragm muscle fiber types.
Waerhaug and Korneliussen 1974	120	Rat (200-300 gm)	Extensor Digitorum Longus (EDL) Soleus (SOL) Semitendinosus (ST) Tibialis Anterior (TA)	Methylene Blue on Teased Fibers	1. Morphological categorization of motor terminals was the same as for the diaphragm (79). 2. Ending categories are somewhat muscle specific. a. EDL and ST are approximately 50% type A. b. SOL is about 89% type B. c. TA has about 74% type A in the superficial part. d. TA has 65% type C in the deep part. 3. Endplates are fiber type specific. a. White fibers (FG) had type A. b. Red fibers (FOG) had type C. c. Intermediate fibers (FG) had type B.
Ip 1974	71	Rat (150-250 gm)	Soleus	AChE, Ag, and AChE-Ag Combined Stains on Frozen Sections	1. Endplates in all muscles were found to have varied staining properties. 2. Endplates innervated by the same terminal axon but on different fibers showed stain variations. 3. No relationship existed between endplate-staining traits and endplate or muscle fiber size.
Tweedle and Stephens 1981	117	Rat (50-250 gm)	Adductor Longus Soleus Plantaris Rectus Femoris	Bielchowsky-Cholinesterase Combined Stain on Frozen Sections	1. Terminal motor endings are more "complex" in mature muscle. 2. Terminal motor endings in slow-twitch muscle are more "complex", and have higher percentages of accessory and double endings that fast-twitch muscles taken from post-pubertal animals.

each class. His "terminaisons en ligne" were endings adapted to animals needing quick, violent muscular activities, while "terminaisons en grappe" were seen to reflect requirements for finely co-ordinated muscular activity. In this light Cole saw the latter as a primitive, less highly differentiated type of nerve terminal which was most related to tonic functions.

In further restricting his observations Cole pointed out the high degree of specificity and functional adaptation of endings. His comparison of neuromuscular contact areas suggested, for example, that in non-flight birds the value for this parameter was roughly one-half that of their flying counterparts. Apparently coincident with the loss of the capacity for the quick, forceful movements of flight was the loss of a significant amount of neuromuscular interaction area. Leg muscle comparisons from both flight and non-flight birds to the back muscles of flight birds were not different--a fact which Cole surmised to be indicative of the lack of species alteration. He concluded that these and other similar observations were proof for functional adaptation of endings. Likewise the lack of structural differences of endplates taken from the selected muscles (muscles which "were closely related in" function and histological traits) of each species was considered indicative of functional adaptation.

In 1957 Cole (31) focused his investigation on differences which might exist between muscles of different functions taken from the same animal. On the basis of similar morphological characteristics of motor endings taken from seven muscles of animals of over 150 grams Cole was able to delineate 3 specific groups. Muscles capable of highly skilled movements were found to have a constant pattern of compact "terminaisons en plaque" and narrow muscle fibers. The diaphragm, a relatively tonically active respiratory muscle, was found to have a wide variety of ending types--both "terminaisons en grappe" and "en plaque" with the former appearing considerably more often. The postural and appendage muscles comprised the third grouping and featured large loose "terminaisons en plaque". Cole considered these findings supportive of his "functional adaptation" theory and further suggested that the "terminaisons en grappe" might "account for the absence of fatigue" seen in the diaphragm muscle.

Hess (66) noted that in mammalian extraocular muscles fast (white) fibers had single endings of the "en plaque" type while the preponderance of terminals in slow (red) fibers was the "terminaison en grappe".

Citing this and the lack of investigation of specificity of motor terminals as they relate to fiber types, Ogata (90) conducted a histochemical study with mice using a double staining method of succinic dehydrogenase (SDH) and cholinesterase (Ch). While differences in size and structure were noted, no marked differences in cholinesterase activity were seen. All endings were of the "en plaque" type for the gastrocnemius and adductor magnus muscles. Unfortunately, with Ogata's technique no indication of neural input was obtained. He did suggest that the differences in size and complexity might be attributed to physiological characteristics or muscle fiber size.

Korneliussen and Waerhang (79) and Waerhang and Korneliussen (120) categorized terminal endings in diaphragm and hindlimb muscles of rats according to size and specific characteristics which these terminals displayed within the endplate region (primarily intra-endplate branching and bouton shape patterns). While the methylene blue stain used presented problems, their results did indicate both muscle and fiber type specificity for endings. Type A terminals appeared related to "white" fibers, type B terminals were associated mainly with "intermediate" fibers, and type C arborizations innervated "red" fibers. Muscle fiber types were determined by a fat stain (78) and were well correlated with Ariano's classifications (3). Further the authors hypothesized that "each of the types of motor nerve terminals may innervate one of the three functional types of motor units defined by Burke et al. (20,21)". Finally, all endings were defined as "en plaque".

As their categorization system and results appeared to be corroborated by earlier investigations (2,28,52,53), it was suggested that the pattern of innervation (size and number of terminal branches or swellings, and overall endplate size) was influential in both total amount of transmitter released and area available on the postsynaptic membrane for reception of transmitter. Presynaptic morphological traits were thought to possibly be involved with regard to the physiological differences of the various mammalian twitch fibers.

Further examination of the postsynaptic membrane and endplate area by Ip (71) further emphasized the specific and dynamic nature of the postsynaptic ending. The finding of both pale- and strong-staining endplates in the same field, and in some cases associated with branches from the same terminal axon, suggested a non-uniform structure in normal muscle--one which was either related to specific fiber characteristics or one which was indicative of a transitional state. It was suggested that a dynamic subneural apparatus could account for their differential staining characteristics and the possible fiber-type relationship.

Comparison of prepubertal and postpubertal rat motor endings by Tweedle and Stephens (117) substantiated many of the previous findings. In mature animals terminal motor endings were categorized and it was found that primarily slow-twitch muscles had a different innervation pattern than those fast-twitch muscles examined. Generally slow-twitch muscles had terminals which were more complex in that they had either numerous branches entering one endplate region or several rather diffuse endplate areas associated with a branched terminal axon. This complexity was not visible in fast-twitch muscles nor in muscle taken from animals under 5 weeks of age. This elaboration of motor endings appeared, in some cases, to be related to activity level while in others, was surmised to be a function of a maturation or ageing process.

<u>Junction Size</u>--As with morphological characteristics, endplates or terminal motor ending size may be related to muscle fiber size or type. Numerous studies (2,53,56,59,71,79,94,110,116) have reported a direct relationship between endplate diameter/length and muscle fiber diameter. Ogata (90), in addition, has reported a possible relationship between fiber type and endplate size. His white fibers had endplates on average 23μ, while red and intermediate fibers had endplates measuring an average 14μ and 20μ respectively.

Similarly, measurement of terminal ending diameters in the three types of endings from the work of Korneliussen and Waerhang (79,120) indicated A-type terminals innervated thicker fibers while B-type and C-type innervated intermediate and thin fibers respectively. As with endplate data, that on endings appears to indicate that the size of the terminals is approximately proportional to the width of their muscle fibers. A correlation between fiber type and ending size was not directly available, though considered viable by the investigators.

Dias (36) using intravital staining with methylene blue and a grid-area measure instead of diameter indicated an extremely wide range of ending areas was present in all muscles of the rabbit studied. He found the slow soleus had mean surface areas for motor endings which were significantly larger than either of the predominantly fast-twitch gastrocnemius or flexor digitorum longus muscles and consequently suggested a relationship existed between speed of contraction of a muscle and the size of its endings. In the soleus the large size of the motor ending was related to its function. It was proposed that in this postural, continuously active muscle the large size ending either facilitated the release of neurotransmitter or was involved in the trophic regulation of speed of contraction of the muscle.

Electronmicroscopic Investigations (see Table 2.2)--The electronmicroscopic investigations of myoneural junctions by Ogata's group (87,91,92) revealed the fine structural differences of motor endings and endplates taken from "red", "white", and "intermediate" muscle fibers. The differentiation of muscle fiber type by identification of the size and composition of mitochondrial chains among the interfibrillar spaces, deposition of mitochondria beneath the sarcolemma, and the size and shape of the bracelet-like mitochondria around the myofibril at each I-band enabled these investigators to firmly establish the specific characteristics for neuromuscular junctions of each fiber type.

Table 2.2. Morphological Specificity of Terminal Endings/Endplates -- Electronmicroscopic Results

Investigator(s)	Reference	Animal Model	Muscle Used	Generalized Findings
Ogata, Hondo and Seito 1967	91	Rat (200-300 gm)	Intercostal	1. White fibers had large endplates with well developed and numerous junctional folds. Folds were deep, extensively branched and anastomosed. Mitochondria in junctional sarcoplasm were few. Complex appearance. 2. Red fibers had small endplates with poorly developed junctional folds. Large areas existed between folds and terminals which were filled with mitochondria aggregations. Folds were shallow, with minor branching and anastomoses. Simple appearance. 3. Intermediate fiber had endplates of intermediate characteristics.
Murata and Ogata 1969	87	Human	Intercostal	1. Results were in agreement with previous work (91). 2. White fibers also had large, single membrane vesicles located at the bottom and sides of the folds. 3. Red fibers had similar vesicles but only at the bottom of the junctional folds and in the interfibrillar space. 4. Intermediate fibers had various sized vesicles around the folds.
Ogata and Murata 1969	92	Rat (200-300 gm)	Extensor Digitorum Longus	1. Results were in agreement with previous work (87,91).
Duchen 1970	40	Mouse (30 gm)	Soleus Gastrocnemius (superficial)	1. Gastrocnemius muscle had endplates which were more developed (folds per μm synaptic membrane = 2.6; mean depth = 0.8±0.1 μm; and proportion of folds branched = 45%). Mitochondria were inconspicuous. 2. Soleus muscle had endplates with fewer (folds per μm synaptic membrane = 1.3) and shallower (mean depth = 0.5±0.1 μm) folds. Proportion of branching was 16%. Extensive aggregations of long branching mitochondria were present.

Table 2.2.-- Continued

Investigator(s)	Reference	Animal Model	Muscle Used	Generalized Findings
Padykula and Gauthier 1970	93	Rat (200-300 gm)	Diaphragm	1. Endings on white fibers were long and flat with numerous, tightly packed axoplasmic vesicles and slender mitochondria. Endplates associated with these terminals were shallow with numerous junctional folds. Folds were long, closely packed and relatively straight. Sarcoplasm between the base of the folds and the myofibrils was sparse. Mitochondria exist near/between areas of neuromuscular contact only. 2. Red fiber endings were small and elliptical with loosely packed vesicles and filamentous, well developed mitochondria. Endplates here displayed short, curved and fewer junctional folds which were irregulary spaced. Extensive sarcoplasmic development was present between fold bases and myofibrils. Large numbers of well developed mitochondria were present. 3. Intermediate fiber terminals were the longest and deepest. Junctional folds also were deeper but farther apart than those of other fiber-type endings. Other characteristics were "intermediate".
Santa and Engel 1973	109	Rat (180-200 gm)	Soleus Gastrocnemius (superficial)	1. Endings on white fibers were generally smaller than those on other fibers but not significantly. These endings had larger but fewer synaptic vesicles. White fiber endplates had a large presynaptic-to-postsynaptic membrane length. 2. Red fiber terminals were largest in overall area with relatively large numbers but smaller axoplasmic vesicles. 3. Intermediate fiber terminals were intermediate in overall area and had significantly more but smaller vesicles than white muscle terminals.
Ellisman, Rash, Staehelin and Porter 1976	42	Rat (200-400 gm)	Soleus Extensor Digitorum Longus	1. Results generally confirmed those of previous investigations (87,91,92, 93) done at relatively low magnification. 2. No differences were observed between the soleus and extensor digitorum longus neuromuscular junctions at the membrane molecular level.

White fiber neuromuscular junctions were characterized as being the largest of the three types and the most complex. They displayed an extensive array of parallel, yet well-branched and anastomosed, junctional folds. These deep folds were separated from the myofibrils at their bases by scant sarcoplasm. Mitochondria at the junction were few and poorly developed. Red-fiber myoneural junctions, while smaller and simpler, displayed well-developed mitochondria both between folds and at their bases where, in company with nuclei, Golgi apparatus, and a large sarcoplasmic area, they provided a large space between the folds and the myofibrils. Intermediate-fiber synapses showed characteristics generally between those of the red and white junctions.

The functional significance of these traits was thought by these investigators to be related to neuromuscular transmission. The wide junctional area, and characteristic fold pattern of the white fiber synapse was advocated as a mechanism for rapid transmission of impulses to the fast-contracting fiber. Such rapid transmission is not necessary in the slow-contracting fiber; consequently, the ending/endplate structure is smaller with less total surface area.

Padykula and Gauthier (93) also suggested that the extensive surface area offered at the white neuromuscular junction could be related to supplying greater amounts of acetylcholine or some other trophic substance for in addition to the folding membrane network they found in white-muscle junctions significantly more sarcoplasmic vesicles, axoplasmic vesicles, and intramitochondrial granules.

Santa and Engel (109) indicated that the postsynaptic receptor sites had a concentration approximately 20% higher in white and intermediate fibers than in red. In addition the synaptic vesicle diameter was slightly smaller in red and intermediate fibers while the synaptic vesicle count per unit nerve terminal area was larger. Coupled with mean postsynaptic-to-presynaptic membrane length ratios which indicated white fiber endplates are more complex, this would appear to support the contention that the white fiber terminal and endplate are more suited to rapid, phasic impulse transmission.

Morphological Age-Related Changes of Terminal Endings/Endplates

Histological/Histochemical Light Microscopic Studies (see Table 2.3)--Cole's (31) assertion that compact "terminaisons en plaque" were immature endings characteristic of young animals while loose "terminaisons en plaque" or "terminaisons en grappe" were more specific to older animals led to the assumption that there were age-related morphological variations in motor endings.

Baker and Ip (6) investigated these age-related changes and the possible mechanism by which they were brought about. It was their hypothesis that terminals had "a limited life-span" and that they were "periodically replaced in normal muscle by collateral regeneration" once that life-span had expired. The mechanism they advocated for this turnover was that either a new endplate innervated by a sprout from the degenerating endplate's terminal axon was formed on the muscle fiber or that the established endplate received a new ending from a sprout of the parent axon. The former case was thought to account for the presence of the observed "double endings" while both mechanisms accounted for the "accessory endings" which they saw. As an addendum they suggested that

Table 2.3. Age-Related Morphological Changes in Terminal Endings/Endplates -- Light Microscopic Results

Investigator(s)	Reference	Animal Model	Muscles Used	Techniques	Generalized Findings
Cole 1947	30	Rat (approximately 35-75 days)	Intercostals (2nd, 3rd, 4th) "Deep spiny dorsals"	Modified Ranvier Gold Chloride Stain on Teased Fibers	1. No relationship existed between rat weight and endplate width. 2. No significant variation in width or shape of endplate from muscle to muscle or animal to animal, although an increase in endplate size with age was indicated. 3. Double and accessory endings were visible.
Cole 1957	31	Rat (approximately 21-35 days and 42-77 days)	Intercostals Paravertebrals Tongue Foot Interossei Diaphragm Rectus Femoris Gastrocnemius	Modified Ranvier Gold Chloride Stain on Teased Fibers	1. Endplates differ in size and morphology when age is considered. 2. In young (small) animals no "terminaisons en grappe" were present and compact "terminaisons en plaque" predominated. 3. Older (larger) animals had all types of endings and were more complex. 4. Differentiation of muscle groups was possible in older animals using endplate morphology (see Table 2.1.).
Barker and Ip 1965	6	Cat, Rabbit (not given)	Soleus Interosseous Peroneal	Modified de Castro Silver Stain on Teased Fibers	1. Nodal, preterminal and ultraterminal sprouts were seen in normal muscle. Nodal sprouts occurred twice as often as either of the other two. 2. "Sprouts" were seen in older animals as "accessory endings" and still later as "double endings". 3. In skeletomotor muscle accessories made up approximately 11.3%, sprouts about 8.1%, and normal endings roughly 74.3% of all endings examined. 4. Degenerating endplates and swollen axons were visible, apparently "dying back", among normal endings and endplates. 5. Endplates receiving sprouts and also showing swollen axonal input indicated formation of new junctions by "rejuvenation".

Table 2.3.-- Continued

Investigator(s)	Reference	Animal Model	Muscles Used	Techniques	Generalized Findings
Gutmann and Hanzlikova 1965	56	Rat (10, 150, and 720 days)	Extensor Digitorum Longus Soleus	Modified Koelle and Friedenwald Cholinesterase Stain on Frozen Sections	1. Subneural apparatus changes occurred with aging. Young animals showed rounded, immature endplates, while adult animals had oval, well developed apparatus. Old animals had endplates which showed a wide variety of characteristics (dense vs pale staining, irregular cholinesterase deposition, granular fragmentation and vacuolization. 2. The pattern of innervation changed from a regular zig-zag pattern at the junction of the first and second third of the muscle to one which was irregular and dispersed in old animals. 3. Axon terminals within endplates became thick with age. 4. Endplate sizes increase and differentiate during development.
Tuffery 1971	116	Cat (0.5, 6, 10, 15, 18, and 19 years)	Peroneus Digiti Quinti (PDQ) Soleus (SOL)	Modified de Castro Silver Stain on Teased Fibers	1. Ending/endplate categorization system was established. 2. In PDQ muscle there appeared with advancing age: a. A decrease in "simple" endings. b. An increase in "accessory" endings and sprouts (i.e. complexity). c. A decrease in apparent total degeneration of endplates. d. An increase in proportion of terminals which were bizarre appearing. 3. In SOL muscle there appeared with advancing age similar changes in endplate morphology as were seen in the PDQ. 4. Comparison of young PDQ and SOL muscles indicated: a. SOL endplates were less delicate; smaller with fewer, shorter and coarser terminals; and are found on larger (diameter) fibers. b. SOL endings are less complex. 5. With age: a. In the PDQ and SOL the muscle fiber increases in diameter, the endplate diameter remains constant. b. A decrease in PDQ muscle fiber numbers occurred (SOL not measured).

Table 2.3.-- Continued

Investigator(s)	Reference	Animal Model	Muscles Used	Techniques	Generalized Findings
Pestronk, Drachman and Griffin 1980	94	Rat (60, 300, 540, and 840 days)	Soleus	Cholinesterase Silver Stain on Frozen Sections	1. Young animals had the smallest, simplest endplates with few intra-endplate terminal branch points (2.5 per endplate). 2. Mature animals had the largest endplates with over 5 intra-endplate terminal branch points per endplate. 3. Older animals had small, simple endplates with few intra-endplate terminal branch points (2.8 per endplate). Also seen in old animals was multiple innervation of endplates, collateral sprouting evidence, and preterminal axons innervating up to 10 muscle fibers.
Fagg, Scheff and Cotman 1981	43	Rat (90 and 810 days)	Soleus	Zinc Iodide/ Osmium Tetroxide Stain	1. Mature animals displayed small endplates with a low incidence of sprouting (terminal sprouts = 6±2% and collateral sprouts = 7±2%). Terminal sprouts were short. 2. Aged animals had significantly larger endplates which were more complex. "Accessory" endings and extensive sprouting were/was seen (terminal sprouts = 19±2% and collateral sprouts = 55±10%). Terminal sprouts were shown to be twice as long as in younger animals.

it was possible, though rare, for a new sole-plate structure to be formed on the muscle fiber by a sprout following abandonment of the old endplate. With continuous development and regeneration ongoing some sprouts were thought to end up redundant--these failed to form terminals.

Tuffery (116), while in agreement with the finding of motor endings and endplates undergoing growth and degeneration in normal muscles, rejected the Barker and Ip "replacement hypothesis". She showed that few degenerating endplates received growth configurations and that those which did had growth configurations which were also degenerating. Collateral branching as evidence for replacement was also dismissed as there were insufficient increases in this measure when age was considered. Finally the Barker and Ip hypothesis failed to fully account for the increased complexity of ageing myoneural junctions.

Tuffery proposed a theory of ending/endplate elaboration to account for the changes in innervation pattern seen in ageing animals. It was suggested that this elaboration was brought about by increasing workload which in young and maturing animals was seen as a reflection of increased body weight and animal activity, while in aged animals (senile) was attributable in part to a loss of muscle fibers with compensatory hypertrophy of those remaining. With fiber hypertrophy and no visible sign of increase in endplate size in her study, Tuffery suggested that the elaboration process would provide a means for conveying increased nerve impulses or amounts of trophic substance to the synaptic area. As each "branch" provided additional input, a larger portion of the ending could be activated to compensate for the relative decrease in neuromuscular contact area. The reduction of morphological variance between endings of fast- and slow-twitch muscles with ageing was considered a further characteristic of the ageing myoneural junction.

Gutmann and Hanzlikova (56) also showed myoneural junctions were somewhat transitory and reported a "dedifferentiation" of motor endplates with increasing age as well as some examples of degenerating endplates. The differences in fast- and slow-twitch muscle endplate sizes were seen to disappear with age although muscle fiber diameters remained discrete. In addition denervation-like changes were reported in the pattern of innervation where in animals of advanced age the distribution of endplates became somewhat more random with endplates being found in more distal parts of the muscle. These age-related change in endings/endplates were seen by the authors as closely related to a functional decrease in neuromuscular relations. These alterations were also thought to partly explain changes such as decreased fiber numbers (57,59), reduction of motor unit size (57,59,60), or denervation-like adaptations in aged muscle (56,57).

The findings of Pestronk et al. (94) supports a theory for ending elaboration in mature animals and also Gutmann's "functional denervation" hypothesis for senile animals. The expansion of endplates and elaboration of intra-endplate terminals in their maturing animals and the relative decrease in these parameters in their older animals is indicative of the plasticity of the ageing neuromuscular junction, as is the increased sprouting observed by Fagg et al. (43). Further evidence for this mutability is presented pictorially by Pestronk et al. (94) in the form of increased numbers of branched and double endings, and ultrastructurally by several authors (23,44,52).

Electronmicroscopic Investigations (see Table 2.4)--Fujisawa (47) has suggested that shrinkage, retraction, and eventual dissolution of terminals, as well as sprouting, accounts for synaptic reorganization in normal animals. As evidence he has identified, in the vicinity of totally or partially denervated endplates, preterminal nerve fibers which are normal in appearance. He also stated that there were insufficient mutated nerve fibers in normal muscle to account for degeneration as the prime mode of reorganization.

Cardasis and Padykula (23) have suggested the stimulus for realignment during maturation and ageing was related to changes in speed of transmission of motor units, muscle fiber hypertrophy and/or natural conversion of muscle fiber types as explained by Kugelberg (81,82). They further emphasized the role that a rising workload imposed by continuous body growth plays in muscle activity and synaptic reorganization.

Forced Exercise Effects on Terminal Endings/ Endplates

Scant information is available on the effects of exercise on motor ending/endplate structure. Reed (100) focused on the examination of motor endplate surface area in cholinesterase-succinic dehydrogenase stained sections taken from animals which were sedentary, involved in voluntary exercise, or subjected to a variety of forced exercise programs. It was indicated that running, particularly high-intensity running, results in increased motor endplate surface area as well as accelerating the rate of area increase. It was noted that once the training program was allowed to plateau, endplate size was not maintained. Further, these changes were specific to the soleus muscle. Evaluation of

Table 2.4. Age-Related Morphological Changes in Terminal Endings/Endplates -- Electronmicroscopic Results

Investigator(s)	Reference	Animal Model	Muscles Used	Generalized Findings
Fujisawa 1976	47	Rat (536, 692 and 735 days)	Medial Thigh Muscles	1. Degenerative changes were observed equally in red and white myoneural junctions which were characterized by: a. Indentation of terminal axon axolemmal membrane (could divide ending into lobules). b. The presence of vesicles of various sizes (400-3300 Å). c. Shrinkage of ending with loss of shape and reduction of observed contact area with endplate. d. Vacuolization within the ending.
Cardasis and Padykula 1981	23	Rat (31-260 days)	Soleus (also Gastrocnemius, Extensor Digitorum Longus, and Diaphragm)	1. One third of soleus junctions showed areas of junctional folds which were not covered by axon terminals but associated with redundant basal lamina and Schwann cells. 2. Occasional small axons (sprouts?) appeared near such uninnervated areas. 3. Gastrocnemius myoneural junctions show similar areas though less frequently. 4. Spatial separation of endings and endplates with possible Schwann cell intervention between them was illustrated.
Fahim and Robbins 1981	44	Mouse (210 and 870 days)	Soleus Extensor Digitorum Longus	1. Old terminals, primarily in soleus muscle, showed: a. Loss of synaptic vesicles and decrease in mitochondrial area. b. Increases in microtubules, neurofilaments, smooth endoplasmic reticulum, and areas of uncovered postsynaptic folds. c. Schwann cell intervention between terminal and endplate structures. d. Widened synaptic clefts.

tibialis anterior muscles indicated that there was a trend toward decreased motor endplate size in all groups examined. A possible relationship between muscle fiber types, their conversion due to exercise, and motor endplate size was suggested. Owing to the relatively small number of endplates measured per treatment group the implications of these results should be carefully considered.

Crockett et al. (35) considered the effects of endurance exercise on neuromuscular junction cholinesterase and choline acetyltransferase. While no differences were found in choline acetyltransferase which could be attributed to exercise, distinct differences did exist between the soleus, red vastus, and white vastus of the rat in this parameter suggesting fiber-type specificity for enzyme action. Cholinesterase changes were noted. In untrained animals it appeared that fast-twitch glycolytic fibers had 15% more cholinesterase than fast-twitch oxidative glycolytic fibers. Likewise the latter had 34% more activity than the slow-twitch oxidative fibers. Training affected only the white vastus (fast-twitch glycolytic fibers) muscle--here an increased level of cholinesterase was present. The apparent discrepancies in cholinesterase activity between specific fiber types were seen as compatible with the relative endplate size, degree of ending/endplate development, and the magnitude of the fiber-specific membrane potential.

CHAPTER III

METHODS OF PROCEDURE

The purpose of the current investigation was to determine the effects of both maturation and an exhaustive, endurance-exercise program on the morphology of the motor nerve ending. Specific differences in the innervation pattern and terminal ending structure between selected fast- and slow-twitch muscles also were examined.

Experimental Animals

One hundred normal male albino rats (Sprague-Dawley strain) were obtained in two shipments (Table 3.1) from Hormone Assay, Inc., Chicago, Illinois. Animals were either 30 days or 72 days of age upon arrival. All were allowed a standard period of 12 days for adjustment to laboratory conditions prior to sacrifice or the onset of the respective treatments.

Research Design and Treatment Groups

In accord with the basic objectives of this investigation, treatment groups were designated by both type and duration of activity (Table 3.1). Each animal was randomly assigned to one of the following treatment groups:

1) Control group (CON). The control group received no special treatment. These animals were housed in individual sedentary

Table 3.1. Research Design

		Duration					
	Age At Sacrifice (Weeks)	6	12	16	20	24	28
	Training (Weeks)	0	0	4	8	12	16
Sedentary	N	10	10	10	10	10	9
	Arrival Date	26 Apr 78	26 Apr 78	26 Apr 78	26 Apr 78	27 Mar 78	27 Mar 78
	Sacrifice Date	8 May 78	8 May 78	5 Jun 78	3 Jul 78	3 Jul 78	31 Jul 78
Exercise	N	----	----	10	8	10	8
	Arrival Date	----	----	27 Mar 78	27 Mar 78	27 Mar 78	27 Mar 78
	Sacrifice Date	----	----	8 May 78	5 Jun 78	3 Jul 78	31 Jul 78

cages (24cm x 18 cm x 18cm) throughout the investigation.

2) Endurance-running group (END). The endurance-running rats were housed in voluntary-activity cages for the initial 12-day adjustment period and subsequently in sedentary cages for the duration of the experiment. The END animals were subjected to a previously standardized, progressive program of endurance running (Appendix A).

Training Procedures

The training program was administered once per day, between 1:00 P.M. and 5:00 P.M., five days per week (Monday through Friday). All training was conducted in individual controlled-running wheels (CRW) (121). Training was initiated when the animals were 84 days of age and was continued for 4, 8, 12, or 16 weeks.

The following data were collected during the training and maintenance periods to monitor and document the study and to ensure the END animals were well trained:

1) Weekly records of the body weights of all animals were kept as were daily body weights (pre- and post-exercise) of all animals subjected to forced exercise. Onset-of-treatment and sacrifice weights also were noted (Appendix B).

2) Daily-records were kept of the temperature, barometric pressure, and relative humidity in both the training room and the animal housing quarters (Appendix B).

3) Daily records were kept of CRW master control unit settings used with the END group. These included acceleration time, work time, rest time, number of repetitions per bout, number of bouts, time between bouts, shock level, and expected running speed (Appendix A).

4) Daily records were kept of CRW results for each animal in the END group. These data included total number of revolutions run, percent of expected revolutions, cumulative duration of shock, and percent shock-free time. Data on total meters run (TMR) and percent expected meters (PEM) were calculated for each animal each day. Mean daily values of TMR and PEM for the END rats included in the study appear in Appendix B

Calculation of TMR and PEM provided values which served as the chief criteria in the evaluation and comparison of training performances. Final inclusion of an END animal in the study was based on its ability to exceed 80% of the expected meters to be run and a subjective evaluation of the pattern of its training results. This was necessitated by several problems which arose during course of the study with the CRWs. The effect of "rocking" in the wheels (activation of the revolution counter without actually running a revolution) was thought in some cases to have slightly inflated the training values, specifically TMR and PEM. In addition, internal problems, on occasion, with the master control unit and individual problems with current flow in CRW grids negated the shock-related data. While these problems certainly limit the reproducibility of the study, it should be noted that the training program was very exhaustive and intense. Those animals meeting the selection criteria were well-trained (endurance) subjects.

Animal Care

Throughout the experiment all animals received water and a commercial rat food (Wayne Laboratory Blox) *ad libitum*. Animals in both CON and END groups were handled five days per week. The butcher's paper under the cages and running wheels was changed daily. Additional standardized procedures for daily CRW cleaning were observed and all housing cages were steam cleaned every two weeks.

The animals were maintained in a relatively constant environment with a controlled temperature and humidity. An automatically regulated light sequence was established so that the lights were off between 1:00 P.M. and 1:00 A.M. and on between 1:00 A.M. and 1:00 P.M. This lighting pattern enabled training of the animals during the active phase of their diurnal cycle.

Sacrifice Procedures

Four sacrifices were conducted; one each on the Monday approximately 72 hours following the last treatment of the exercised animals (Table 3.1). All tissues from the animals included in each sacrifice were processed immediately and then stored for simultaneous analysis following the last sacrifice.

A standard sacrifice routine had been developed during the course of previous investigations and was continued without alteration in this study. Likewise an experienced team was retained throughout all sacrifices and tissue processing sessions.

Immediately prior to sacrifices final body weights were recorded and a randomized sacrifice order was established. Subsequently, each animal was killed by a intraperitoneal injection (4 mg/100 gm body weight) of a 6.48% sodium pentobarbital (Nembutal) solution. As soon as the animal was dead, the right hindlimb was skinned and the exposed superficial adductor longus muscle was removed. The superficial posterior crural muscles were exposed by reflecting the overlying tissue, and the gastrocnemius and soleus muscles were removed independently. Finally the rectus femoris muscle was removed.

Immediately after removal from the animal a block of tissue from the belly area of each of the right hindlimb slow-twitch muscles and a similar tissue block from known areas of predominantly fast-twitch fibers in both the right hindlimb gastrocnemius and rectus femoris muscles was mounted on a cork strip using 5% gum tragacanth and subsequently quick frozen in isopentane cooled with liquid nitrogen. Blocks were stored in a freezer at -80°F until sectioned.

An identical procedure for removal of the left hindlimb muscles was carried out. However, prior to further processing the soleus and rectus femoris were utilized as part of an additional study to examine, _in vitro_, the contractile characteristics of these maturing or exercised muscles. In addition, all excised left hindlimb muscles were weighed before placement for fixation in a 10% formal-saline solution with 0.5% dimethyl sulfoxide (DMSO).

This sequence of events (for removal of muscle) has been conducted routinely over the past several years and can be completed within 10 minutes.

Tissue Analysis

Right Leg

Five sets of three serial cross-sections, 10 micra thick, were cut from each muscle using a rotary microtome-cryostat. Each section was identified by muscle, animal number, and date of processing. One section from each set was subjected to one of the following histologic or histochemical procedures: a) Harris's alum Hematoxylin and Eosin (H&E) for demonstration of basic morphological characteristics (83), b) Dubowitz and Brooke's method for demonstration of myosin adenosine triphosphatase (ATPase 9.4) activity for differentiation between fast- and slow-twitch fibers (39), and c) as described by Barka and Anderson using NBT, succinic dehydrogenase (SDH) activity was demonstrated for oxidative capacity indication (5). Sections from all animals of a given sacrifice were processed simultaneously to ensure the use of identical techniques between those animals.

A representative group of 40 muscle fibers from the midportion of each muscle was selected for further study and only the best of the stained sets was used. The relative staining intensities for both ATPase and SDH for each of the 50 fibers was subjectively evaluated, recorded, and used to verify (see Appendix C) previously reported fiber-type compositions for the muscles examined (3,107).

Left Leg

Muscle blocks from the left leg were fixed in 10% formal-saline with 0.5% DMSO for 6 hours, washed in distilled water rinses over 16 hours, and then frozen on metal chucks immersed in liquid nitrogen (only the chuck stem, not the tissue, was immersed).

A minimum of six serial longitudinal sections, 60 to 70 micra thick, were cut from each muscle and placed in a solution of 10% sugar water. These sections were stained using the combined cholinesterase-Bielchowsky method of Gwyn and Heardman for motor endplate-terminal axon morphology (62).

This stain has proven very reliable and has enabled the development of a quantifiable ending classification system (117). In addition it enables evaluation of the majority of endings in a muscle section. Other staining techniques such as the method of Pestronk et al. (94) or Cöers' methylene blue (28) have proven unsatisfactory as they either fail to demonstrate the desired ending structures or are unreliable when used with large groups of animals and sections.

The morphology of the terminal arborization was evaluated by the primary investigator for each muscle examined. Random spot checks by Dr. Charles Tweedle were used to verify the rating system and to ensure consistency in examining such a large number of slides.

A minimum of 100 clearly defined terminal arborizations and endplate structures were categorized as accessory endings, double endings, branched endings, simple endings, multiple endings, double-innervated endings or sprouts (nodal, preterminal, and ultraterminal) (Figures 3.1 and 3.2). An additional division was created by summing the percentages in the accessory, double, branched, and multiple ending categories and is indicative of the percentage of "complex endings" in the tissue examined. Abruptly ending or 'broken' axons were eliminated from any data collection or analysis. Slides with less than 100 endplates were either recut and re-analyzed or eliminated from analyses (if recuts produced less than the specified number of terminal ramifications).

Analyses of Data

Comparative statistical analyses were done using the Mann-Whitney U-test (111). A statistical probability (P) of less than 0.05 was considered to indicate significant difference between means.

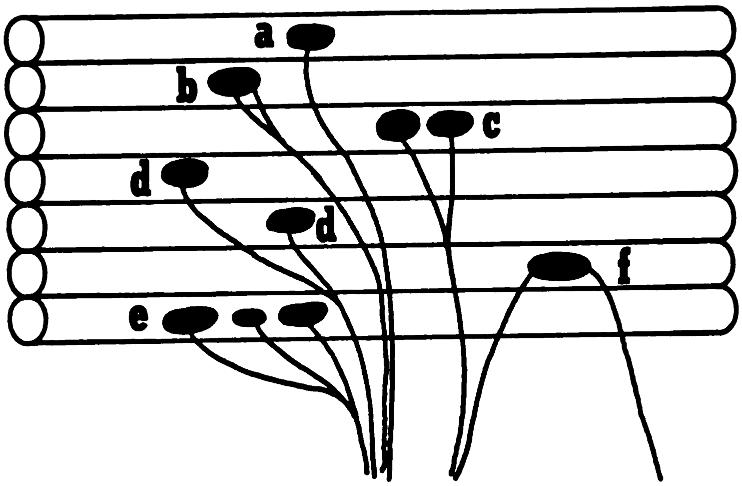


Figure 3.1. Classifications of motor nerve terminals.

a) Simple ending. One axon terminating at one endplate innervating a single muscle fiber (see also Figure 3.2d).

b) Accessory ending. An axon with one or more thin branches originating either from the nodes of Ranvier or at the end of the myelin sheath which inserts into one endplate on a single fiber (see also Figure 3.2c).

c) Double ending. A bifurcated terminal axon which ends in 2 distinct endplates on one muscle fiber (see also Figure 3.2d).

d) Branched ending. A branched terminal axon which culminates in 2 endplates on 2 separate muscle fibers (see also Figure 3.2e).

e) Multiple ending. Three or more branches from a single terminal axon which form more than 2 endplate structures on a single muscle fiber (see also Figure 3.2f)

f) Double-innervated ending. A single endplate structure on one muscle fiber apparently innervated by two distinct terminal axons (these were very rarely seen).

g) Sprouts. Fine unmyelinated fiber(s) originating either from the nodes of Ranvier, the end of the myelin sheath, or the endplate itself and ending in a growth cone (see also Figure 3.2a and 3.2b)

Figure 3.2. Cholinesterase-Bielchowsky stain for morphology of motor nerve endings.

a) Nodal sprout

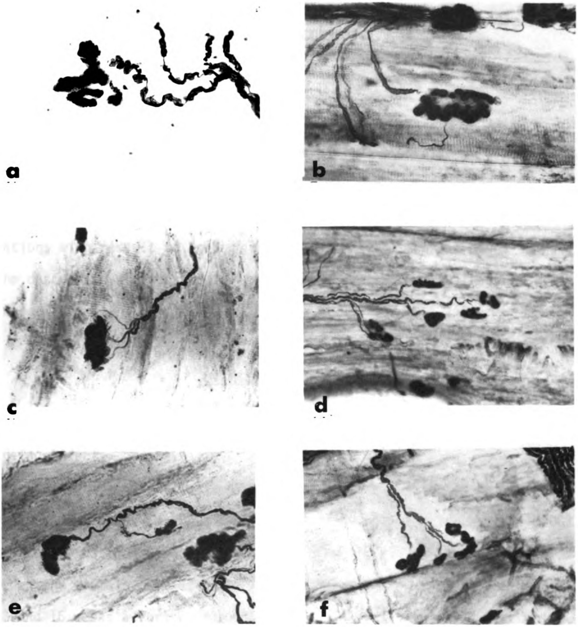
b) Ultraterminal sprout

c) Accessory ending

d) Double and simple endings

e) Branched ending

f) Multiple ending


a
b
c
d
e
f

CHAPTER IV

RESULTS AND DISCUSSION

Results

The results of this investigation will be presented in six main sections corresponding to the terminal nerve ending categories. Each section will be further subdivided to discuss changes in terminal arborizations with respect to ageing, muscle fiber type, and exercise. Finally, the discussion attempts to relate the present findings to those of other investigators.

Accessory Endings

(Figures 4.1 and Tables 4.1 and 4.2)

Ageing Effects

A significant increase in accessory endings was noted between six weeks and 12 weeks in the soleus ($P = 0.001$), adductor longus ($P = 0.001$), gastrocnemius ($P = 0.001$), and rectus femoris ($P = 0.01$) muscles of sedentary animals. In addition in the adductor longus muscle between 12 and 16 weeks a further elevation was visible ($P = 0.025$) after which time no change in mean percentage accessory endings occurred. A similar ageing trend in the soleus was apparent up to 20 weeks ($P = 0.01$). However, a subsequent decrease then was noted in this muscle between

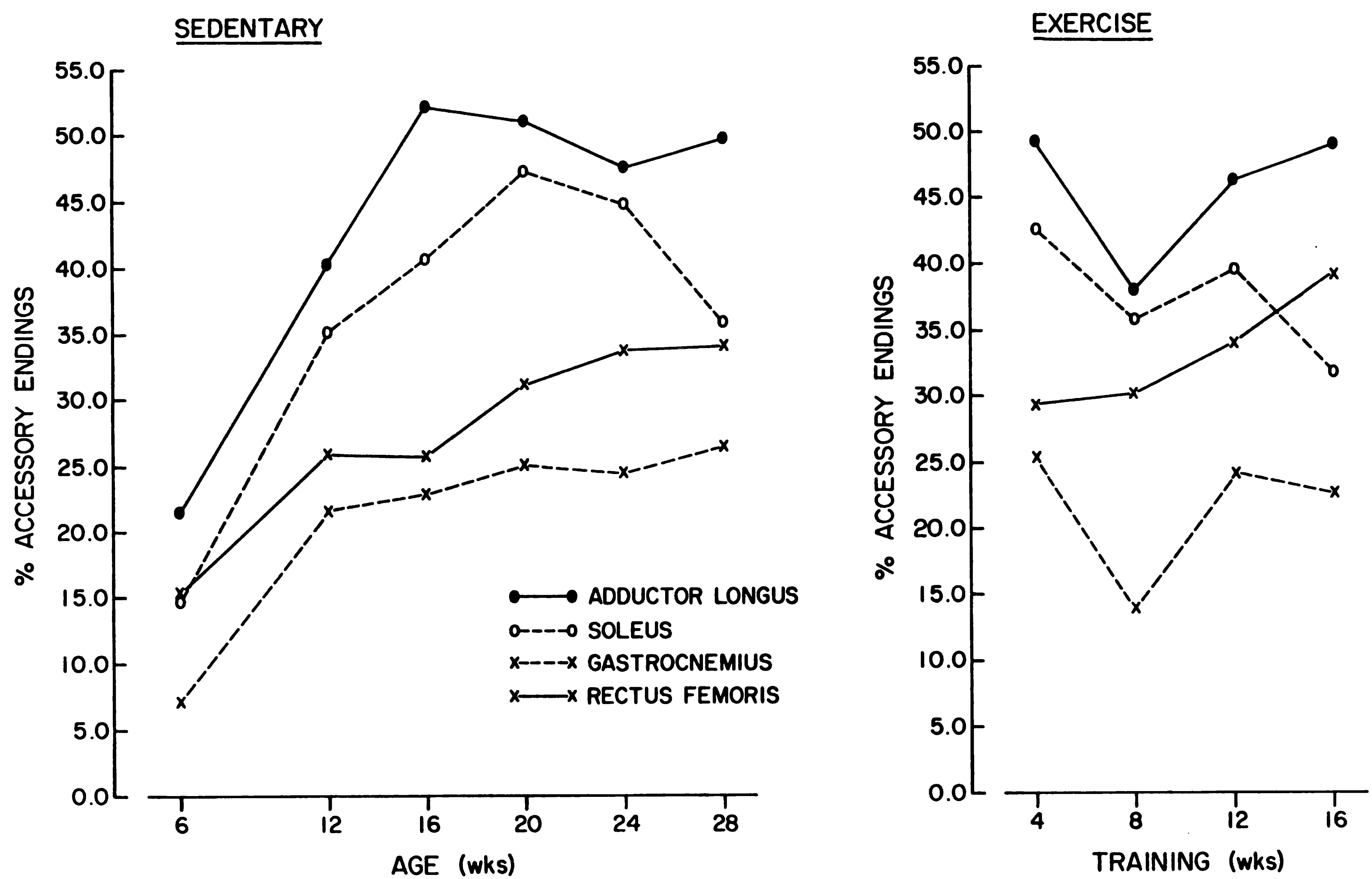


Figure 4.1. Percentage accessory endings in selected muscles of sedentary and exercised rats. (See text for significant differences.)

Table 4.1. Means and Standard Deviations for Terminal Nerve Endings in Sedentary Animals

Muscle	Ending Category	Sacrifice Age: 6 Weeks		12 Weeks		16 Weeks		20 Weeks		24 Weeks		28 Weeks	
		Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
Adductor Longus	Accessory	21.5	9.1	40.3	9.9	52.2	8.5	51.1	4.4	47.5	6.4	49.7	7.8
	Double	2.2	1.4	4.5	2.5	7.8	3.4	8.3	3.5	13.1	4.5	11.0	3.3
	Branched	2.9	1.8	2.7	1.5	2.0	1.5	1.0	1.3	2.1	1.5	1.6	1.3
	Simple	70.6	8.4	51.5	11.0	35.7	11.7	33.7	7.3	34.4	10.8	30.1	7.5
	Multiple	0.1	0.3	0.3	0.5	1.2	1.3	1.7	1.4	2.5	2.4	2.3	1.9
	Sprouts	2.7	1.6	0.7	0.7	1.1	1.1	1.5	1.5	1.4	1.3	1.3	1.3
	Complex	26.7	8.4	47.8	10.8	63.2	11.1	62.9	5.2	65.3	11.6	66.9	7.6
Soleus	Accessory	14.8	5.4	35.1	10.1	40.6	7.9	47.3	7.7	44.8	5.6	35.8	9.9
	Double	1.4	1.0	4.3	1.9	4.8	3.7	10.0	3.4	8.8	2.0	10.2	2.8
	Branched	1.6	2.0	1.3	1.8	0.8	0.8	1.6	1.0	1.3	1.1	2.9	1.6
	Simple	82.1	4.8	59.0	11.0	53.3	9.3	41.0	9.7	44.1	6.6	49.4	13.3
	Multiple	0.0	0.0	0.1	0.3	0.0	0.0	0.1	0.3	0.4	0.8	1.1	1.1
	Sprouts	0.0	0.0	0.1	0.3	0.5	0.5	0.0	0.0	0.6	0.7	0.4	0.5
	Complex	17.8	4.8	40.8	10.7	46.2	9.5	60.0	10.0	55.3	6.7	50.0	12.9
Rectus Femoris	Accessory	15.4	7.2	25.9	6.4	25.8	11.0	31.1	11.0	33.7	7.5	34.0	6.0
	Double	1.1	1.1	0.4	0.5	0.4	0.5	0.1	0.3	0.6	1.0	0.4	0.5
	Branched	2.4	1.8	1.1	1.3	0.4	0.5	0.4	0.7	0.3	0.5	0.8	1.1
	Simple	80.7	7.9	72.0	6.7	73.1	11.0	68.3	11.0	65.3	7.8	64.1	7.5
	Multiple	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Sprouts	0.4	0.5	0.6	0.7	0.3	0.7	0.0	0.0	0.1	0.3	0.1	0.3
	Complex	18.9	7.9	27.4	6.8	26.6	11.0	31.0	11.0	34.6	7.7	35.2	7.2
Gastrocnemius	Accessory	7.1	4.4	21.5	6.8	22.8	9.8	25.0	11.3	24.4	7.9	26.3	6.1
	Double	1.6	1.1	0.9	0.9	0.6	0.8	0.5	0.8	0.6	0.7	1.0	0.9
	Branched	3.6	3.1	2.3	2.0	0.9	1.0	1.5	1.4	1.2	0.9	1.6	1.4
	Simple	86.3	5.2	75.0	7.3	74.9	10.4	71.7	12.5	72.5	8.0	70.2	6.3
	Multiple	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Sprouts	1.4	1.5	0.3	0.5	0.7	0.7	1.1	0.7	1.2	1.0	0.8	0.4
	Complex	12.3	5.4	24.7	7.5	24.3	10.6	27.0	12.0	26.2	7.7	28.9	6.3

Table 4.2. Means and Standard Deviations for Terminal Nerve Endings in Exercised Animals

Muscle	Ending Category	Training Duration: 4 Weeks Mean	4 Weeks S.D.	8 Weeks Mean	8 Weeks S.D.	12 Weeks Mean	12 Weeks S.D.	16 Weeks Mean	16 Weeks S.D.
Adductor Longus	Accessory	49.3	9.5	38.0	9.0	46.3	7.7	49.0	10.5
	Double	6.3	2.9	8.3	3.7	11.9	3.4	9.3	4.2
	Branched	0.7	0.7	2.5	1.4	0.8	0.9	1.5	1.6
	Simple	41.1	8.8	48.6	13.4	36.5	9.9	36.0	12.4
	Multiple	1.1	0.6	1.3	1.5	3.1	3.1	2.3	1.0
	Sprouts	1.4	0.8	1.4	1.8	1.4	1.4	1.8	1.7
	Complex	57.4	8.9	50.0	13.0	62.1	9.9	62.2	12.2
Soleus	Accessory	42.6	9.9	35.8	10.8	39.6	9.2	31.9	4.6
	Double	6.2	1.7	6.3	3.1	9.0	2.5	13.9	3.8
	Branched	1.1	1.2	0.9	0.6	1.4	0.7	3.3	1.7
	Simple	49.8	10.4	55.9	9.0	48.2	11.2	47.4	8.9
	Multiple	0.1	0.3	0.8	0.7	0.2	0.4	1.9	2.0
	Sprouts	0.2	0.4	0.5	0.8	1.5	1.0	1.7	2.5
	Complex	50.0	10.1	43.6	8.6	50.0	11.5	50.9	9.2
Rectus Femoris	Accessory	29.3	11.4	30.1	9.5	34.0	7.8	39.1	16.7
	Double	0.3	0.5	0.1	0.4	0.1	0.3	0.5	0.5
	Branched	0.6	0.7	0.8	1.4	1.0	0.7	1.3	0.9
	Simple	70.9	10.3	68.6	9.2	64.7	7.9	58.4	17.4
	Multiple	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Sprouts	0.0	0.0	0.0	0.0	0.1	0.3	0.1	0.4
	Complex	30.2	11.4	31.0	9.1	35.1	7.7	40.9	16.8
Gastrocnemius	Accessory	25.4	11.5	14.0	7.8	24.1	7.8	22.6	7.7
	Double	1.2	1.5	0.5	0.5	0.5	0.5	0.5	0.8
	Branched	1.7	1.4	1.4	1.3	1.9	1.2	1.9	1.0
	Simple	71.5	12.1	83.4	8.8	72.5	8.3	73.4	8.6
	Multiple	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Sprouts	0.0	0.0	0.8	0.9	0.8	1.0	0.6	0.9
	Complex	28.3	12.2	15.9	8.2	26.5	7.9	25.6	8.6

20 and 28 weeks of age (P = 0.01). After the initial increase in percentage accessory endings in the gastrocnemius and rectus femoris muscles, significant differences occurred in both only between 12 weeks and 28 weeks (gastrocnemius P = 0.05; rectus femoris P = 0.025).

Fiber Type Differences

Significant differences existed between the primarily fast-twitch and slow-twitch muscles at numerous points. The slow-twitch adductor longus displayed more accessory endings than either the fast-twitch gastrocnemius (P = 0.001) or the fast-twitch rectus femoris (P = 0.001) at all ages except at 6 weeks. At that time there was no notable difference between the adductor longus and the rectus femoris muscle. The slow-twitch soleus muscle had more accessory endings than either the gastrocnemius at all ages (P = 0.001) or the rectus femoris at 12 weeks (P = 0.025), 16 weeks (P = 0.01), 20 weeks (P = 0.01) and 24 weeks (P = 0.01) of age. No significant difference was apparent between the soleus and the rectus femoris at either 6 weeks or 28 weeks of age (P = 0.05).

Differences between slow-twitch muscles occurred only at 6 (P = 0.025), 16 (P = 0.01), and 28 (P = 0.01) weeks of age, while in fast-twitch muscles accessory endings appeared significantly more frequently in the rectus femoris at 6 weeks (P = 0.01), 24 weeks (P = 0.01) and 28 weeks (P = 0.025).

Exercise Effects

No significant differences were seen in the comparison of sedentary animal muscles with those same taken from chronically exercised animals except after eight weeks of training. At that point significant decreases were apparent in the numbers of accessory endings found in the adductor longus (P = 0.002) and soleus (P = 0.05) muscles of the trained animals. A similar decrease, though non-significant (P = 0.10), was present in the gastrocnemius muscle.

Double Endings

(Figure 4.2 and Tables 4.1 and 4.2)

Ageing Effects

An ageing trend was seen in the double endings found in slow-twitch muscles. In the adductor longus significant increases in percentage double endings were seen up to 24 weeks of age. These significant differences were noted between 6 and 16 weeks (P = 0.01), 12 and 16 weeks (P = 0.05) and 20-24 weeks (P = 0.02). The soleus muscle was found to contain increasing numbers of double endings up to 20 weeks of age, with major increases occurring between 6 weeks and 12 weeks (P = 0.001) and between 16 weeks and 20 weeks (P = 0.01). No ageing trend was seen in fast-twitch muscles which consistently displayed less than 2.0% double endings.

Fiber Type Differences

No differences existed at six weeks of age between fast-twitch and slow-twitch muscle terminal arborizations. One percent to 2.2% of the endings seen at this point were doubles. At every point thereafter slow-twitch muscles (adductor longus and soleus) displayed more double endings than fast-twitch muscles (P = 0.001).

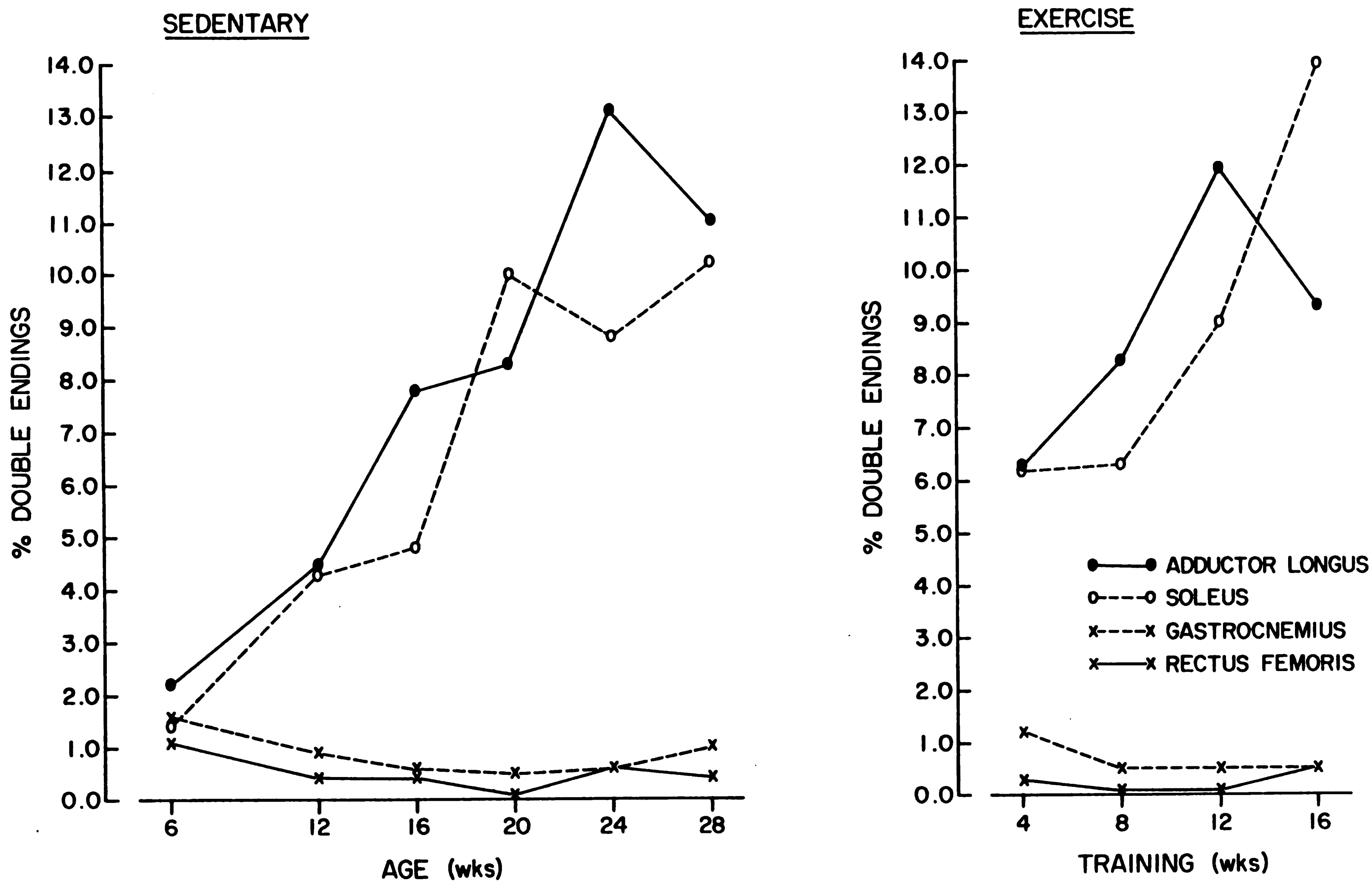


Figure 4.2. Percentage double endings in selected muscles of sedentary and exercised rats. (See text for significant differences.)

At 16 weeks (P = 0.05) and 24 weeks (P = 0.01) differences existed in this category between the adductor longus and soleus muscles. In both instances more doubles were found in the adductor longus. No other 'type' differences were noted.

Exercise Effects

Exercise apparently had very little effect on double endings in these selected muscles. Only in the soleus after 16 weeks of intense exercise was a change seen--an increase in double endings (P = 0.02).

Branched Endings

(Figure 4.3 and Tables 4.1 and 4.2)

Ageing Effects

Less than 4% of all endings seen were branched and following puberty, with rare exceptions, no more than 2.5% could be placed in this category. In the adductor longus muscle no changes could be detected between 6 and 12 weeks of age, nor after 20 weeks of age. A significant decrease in branched endings was detected, however, between 12 weeks and 20 weeks of age in the adductor longus (P = 0.05). The soleus muscle displayed a consistent percentage branched endings of approximately 1.5% until between 24 weeks and 28 weeks of age, at which time the increase to 2.9% was found to be significant (P = 0.05). In the fast-twitch muscles, an ageing trend was apparent in that both muscles showed decreased percentages between 6 and 16 weeks (P = 0.02 for the gastrocnemius and P = 0.002 for the rectus femoris) followed by a levelling-off phase during which the branching of endings appeared constant.

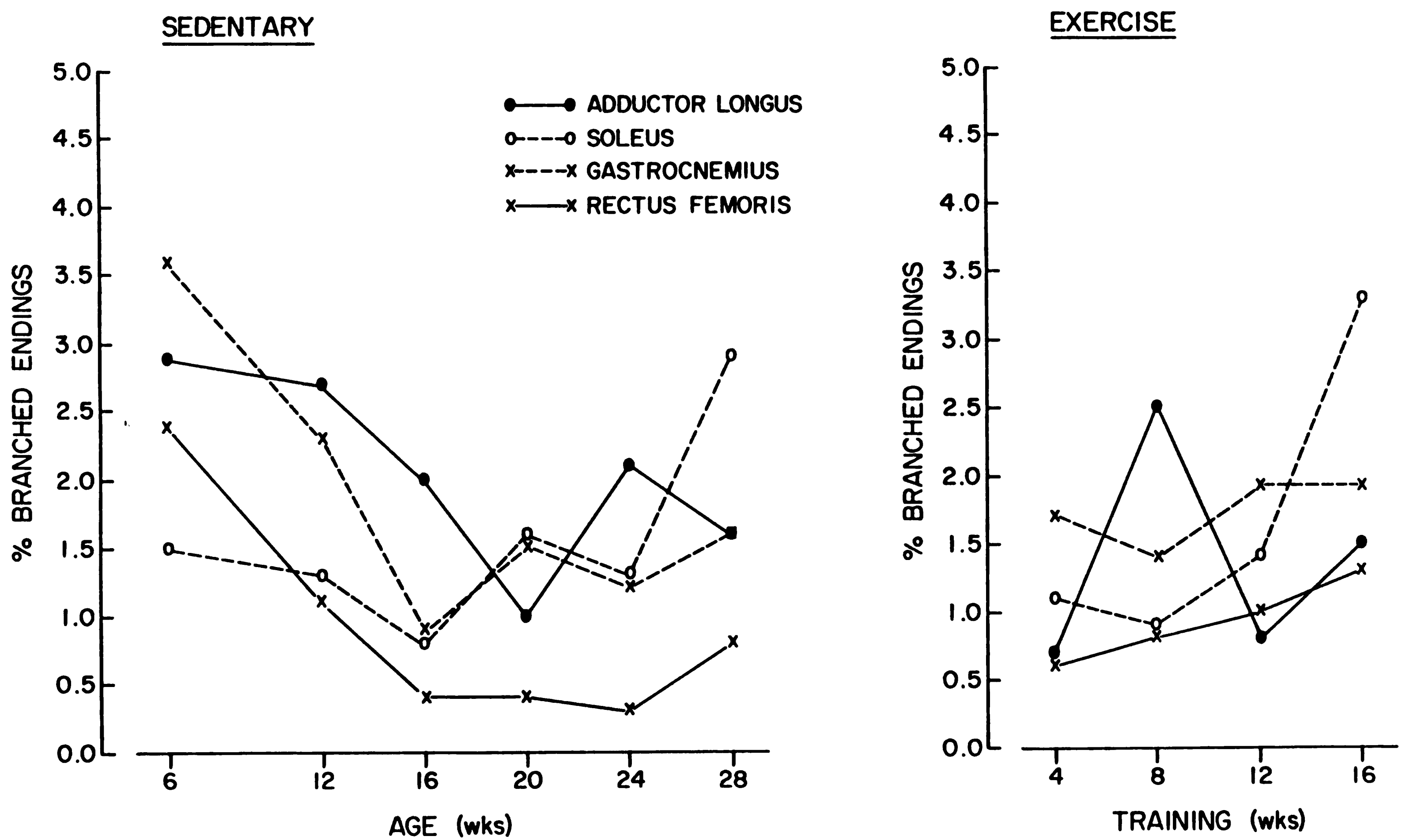


Figure 4.3. Percentage branched endings in selected muscles of sedentary and exercised rats. (See text for significant differences.)

It seemed that branched endings in sedentary animals decreased post-puberty to a base level which was maintained.

Fiber Type Differences

Differences between slow- and fast-twitch muscles were not as readily visible in this ending category, probably due to the relatively few branched endings seen. Neither the adductor longus nor the soleus displayed any difference at any time in terms of branched endings from the gastrocnemius muscle. Differences did exist which were significant between the soleus and rectus femoris at 20 weeks ($P = 0.02$), 24 weeks ($P = 0.05$), and 28 weeks ($P = 0.02$) as well as between the adductor longus and rectus femoris at 12 weeks ($P = 0.05$), 16 weeks ($P = 0.05$), and 24 ($P = 0.02$) weeks of age. In all cases the rectus femoris displayed less branching.

In addition, the soleus was found to differ significantly from the adductor longus only at 12 weeks of age ($P = 0.05$). No major detectable changes could be found between fast-twitch muscles except at 24 weeks of age when the gastrocnemius had more branched endings ($P = 0.05$).

Exercise Effects

As with the two previous categories, it appeared that exercise had minimal effect on motor endplate morphology. Only in the adductor longus was a significant change seen. It appeared that in this muscle, exercise produced an increase in branched endings after 8 weeks of endurance training ($P = 0.05$) and a decrease in percentage branched endings after 4 weeks and 12 weeks ($P = 0.05$).

Simple Endings

(Figure 4.4 and Tables 4.1 and 4.2)

Ageing Effects

Apparent pre-pubertal to post-pubertal (6 weeks to 12 weeks) decreases were seen in all muscles with those changes being significant at P = 0.001 in the adductor longus, soleus, and gastrocnemius and at P = 0.025 in the rectus femoris. This decrease in simplicity of terminal arborizations continued in the adductor longus to 16 weeks (P = 0.05) and in the soleus to 20 weeks (P = 0.01) after which no further significant changes were noted. The gastrocnemius and rectus femoris muscles appeared to "plateau" after 12 weeks of age.

Fiber Type Differences

Muscles defined as primarily slow-twitch had significantly fewer simple endings at all points in time except at 6 weeks when the soleus and rectus femoris were similar. Comparisons between the adductor longus and gastrocnemius were significant at P = 0.001, as were those between the adductor longus and rectus femoris muscles after 12 weeks of age. The rectus femoris versus adductor longus comparison at 6 weeks was significant at P = 0.01. The soleus muscle was found to differ (P = 0.001) at 16 weeks, 20 weeks, and 24 weeks from both the rectus femoris and gastrocnemius. At 12 and 28 weeks of age, the level of significance was P = 0.01 for the soleus versus gastrocnemius and soleus versus rectus femoris comparisons, while the remaining comparison was significant at P = 0.05.

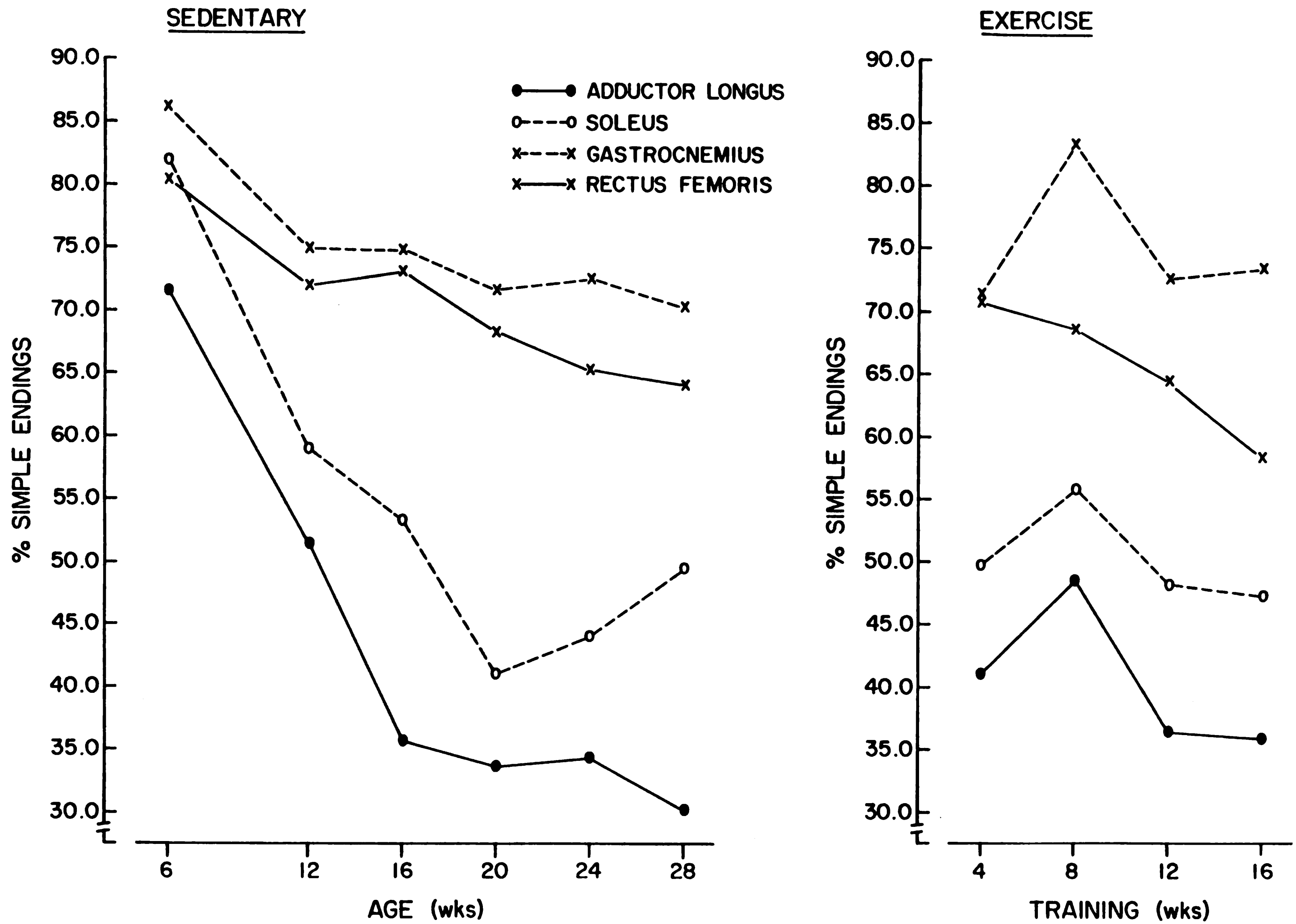


Figure 4.4. Percentage simple endings in selected muscles of sedentary and exercised rats. (See text for significant differences.)

The adductor longus was found to have significantly fewer simple endings than the soleus muscle at 6 (P = 0.001), 16 (P = 0.01), 24 (P = 0.025), and 28 (P = 0.01) weeks. The only differences between fast-twitch muscles were noted at 24 weeks of age (P = 0.05). At all points, however, the rectus femoris displayed a lower percentage simple endings than did the gastrocnemius muscle.

Exercise Effects

Only in the adductor longus and soleus muscles did the exercise program appear to elicit changes and only then after 8 weeks of training. Increases in percentage simple endings at this point were observed (P = 0.02). No other changes were noted.

Sprouts and Multiple Endings

(Figures 4.5 and 4.6 and Tables 4.1 and 4.2)

Ageing Effects

A very low level of sprouting was visible in the muscles during the study. It appeared in less than 3% of the terminal motor nerves and their endings. The decrease seen between 6 weeks and 12 weeks of age in the adductor longus was found to be noteworthy (P = 0.01). While not significant, a pattern appeared which indicated that nodal sprouts were the predominant type. They generally occurred twice as often as preterminal and ultraterminal sprouts combined.

Likewise, multiple endings were relatively rare. Less than 3% were seen in slow-twitch muscles and none were seen in either of the fast-twitch

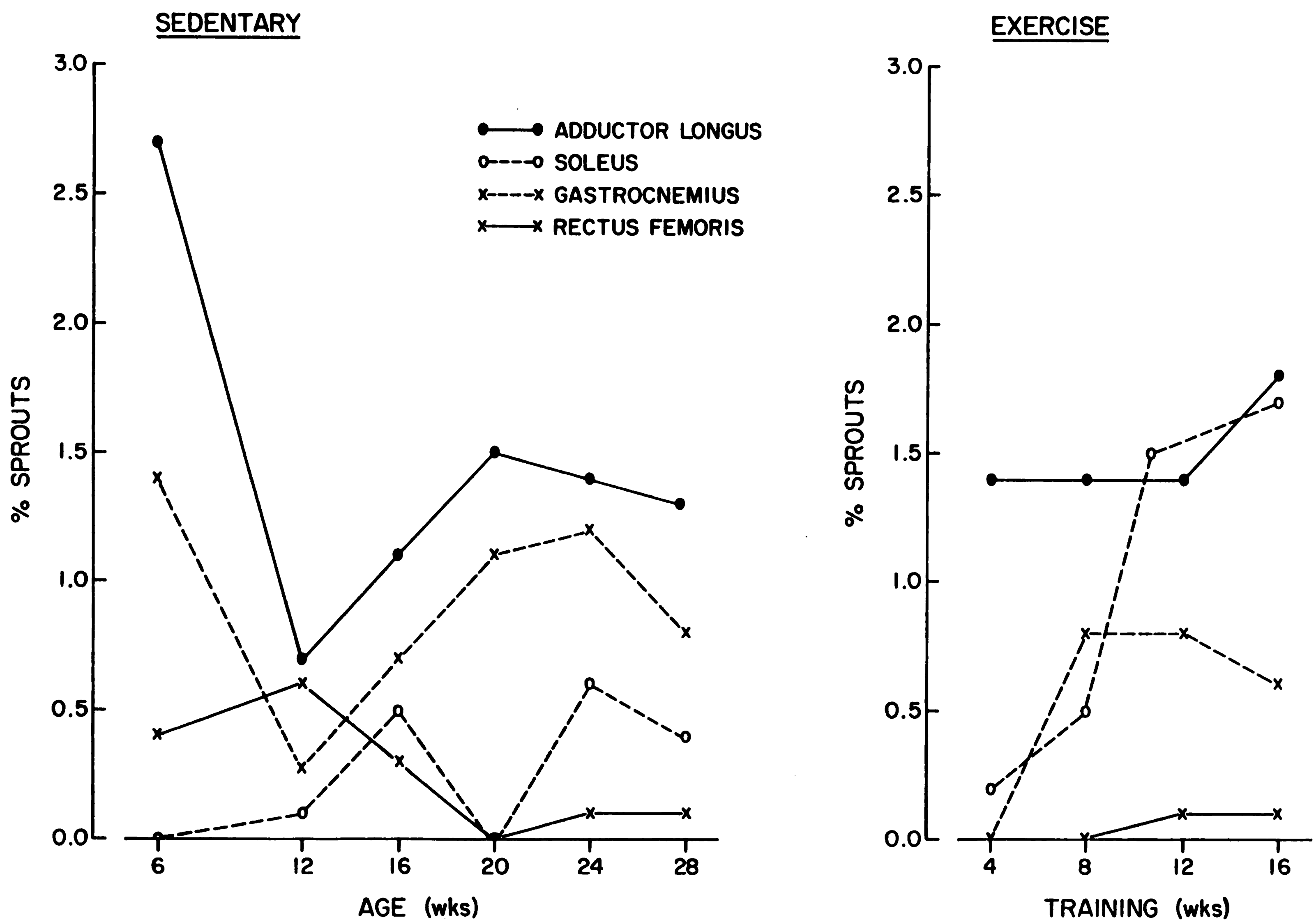


Figure 4.5. Percentage sprouts in selected muscles of sedentary and exercised rats. (See text for significant differences.)

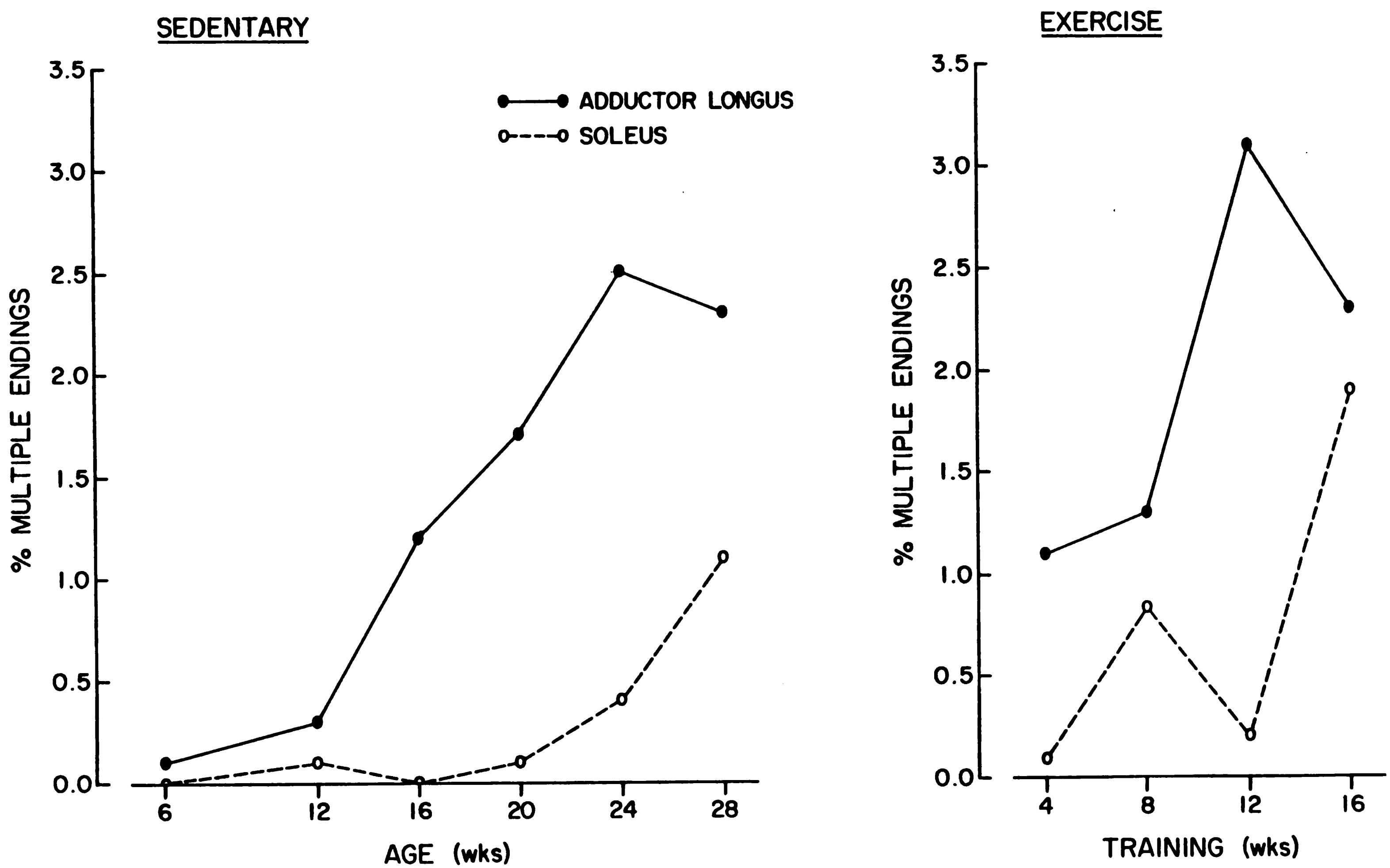


Figure 4.6. Percentage multiple endings in selected muscles of sedentary and exercised rats. (See text for significant differences.)

muscles. Only between 12 and 16 weeks ($P = 0.05$) in the adductor longus was a change noted.

Fiber Type Differences

Unlike the other categories examined, the sprouts category displayed no consistent pattern in terms of differences in endings associated with fiber types. No significant differences were apparent at any point between the soleus and the rectus femoris or between the adductor longus and the gastrocnemius ($P = 0.05$). The adductor longus did appear to have a larger percentage sprouts than the rectus femoris at 6 ($P = 0.02$), 20 ($P = 0.05$), 24 ($P = 0.02$), and 28 ($P = 0.05$) weeks of age. Of interest was the difference between the soleus and gastrocnemius, which while significant at only six weeks ($P = 0.02$) and 20 weeks ($P = 0.002$), did indicate that the fast-twitch muscle had more sprouting than the slow-twitch muscle. The gastrocnemius showed more sprouting than the rectus femoris after 20 weeks of age ($P = 0.02$). The value of these relationships should be carefully considered owing to the relatively small percentage sprouts present.

As no multiples were seen in fast-twitch muscles, significant differences existed between those and slow-twitch muscles at all ages. The only exception was in the soleus versus fast-twitch muscle comparisons at 6 and 16 weeks.

Exercise Effects

Exercise had an effect on only the gastrocnemius sprouts after four weeks of exercise, and then it appeared to bring about somewhat of a decrease in sprouting (P = 0.05). No other changes were affected by chronic training in either sprouts or multiple endings.

Complex Endings

(Figure 4.7 and Tables 4.1 and 4.2)

Ageing Effects

Complex endings appear, at this point in time, to be the best overall indication of synaptic reorganization through increased complexity in the maturing animals. All sedentary rats displayed increased complexity between six weeks (pre-pubertal) and 12 weeks (post-pubertal) of age. These changes were significant in the adductor longus, soleus, and gastrocnemius at P = 0.001 as well as in the rectus femoris at P = 0.025. Whereas complexity appeared to plateau after this age in the primarily fast-twitch muscles, it continued to increase in the adductor longus to 16 weeks (P = 0.01) and in the soleus to 20 weeks (P = 0.01) prior to levelling off.

Fiber Types Differences

At six weeks of age there was no difference between the numbers of complex endings in the rectus femoris and in the soleus, while at 12 weeks of age this same comparison was found to be significant (P = 0.01). The soleus also had a larger percentage complex endings than the gastrocnemius at both six and 12 weeks (P = 0.01). After 12 weeks of age, the soleus muscle was more elaborate than either of the fast-twitch muscles (P = 0.001). This same level of significance was detected in all adductor

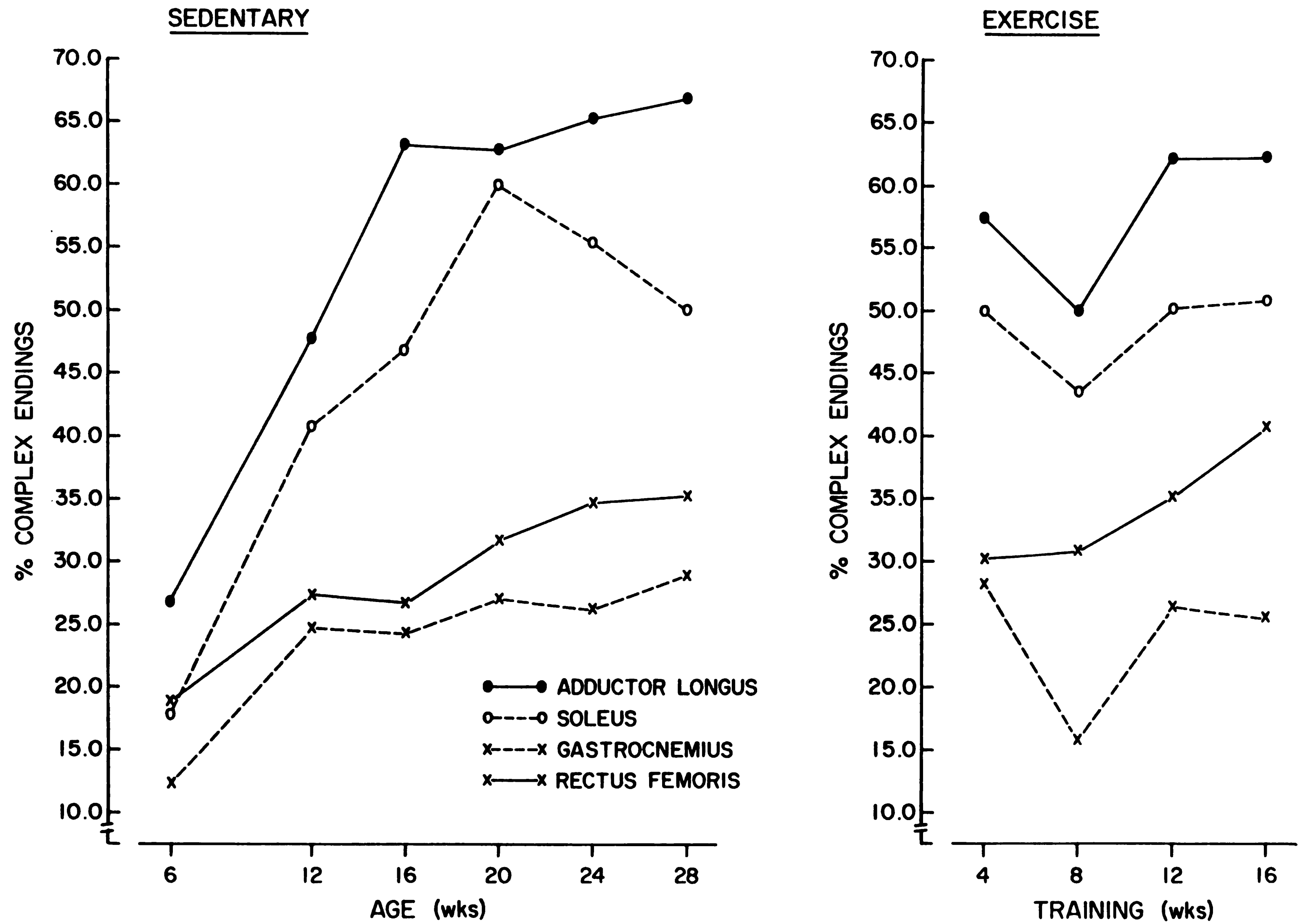


Figure 4.7. Percentage complex endings in selected muscles of sedentary and exercised rats. (See text for significant differences.)

longus versus fast-twitch muscle comparisons except the six-week comparison with the rectus femoris (P = 0.05).

The adductor longus also displayed more complex endings than the soleus at all ages with those at 6 (P = 0.01), 16 (P = 0.01), 24 (P = 0.05), and 28 (P = 0.01) weeks of age being significantly higher. In comparisons between fast-twitch muscles, the rectus femoris was found to be more complex than the gastrocnemius at all ages--those values at 6 (P = 0.05), 20 (P = 0.05), 24 (P = 0.025), and 28 (P = 0.01) weeks being significant.

Exercise Effects

Once again the exercise program had little, if any, effect. Only in the slow-twitch muscles after eight weeks of training could significant differences be detected in the adductor longus (P = 0.05) or the soleus (P = 0.02) muscles. In both instances, a decrease in complexity at the ending was visible.

Discussion

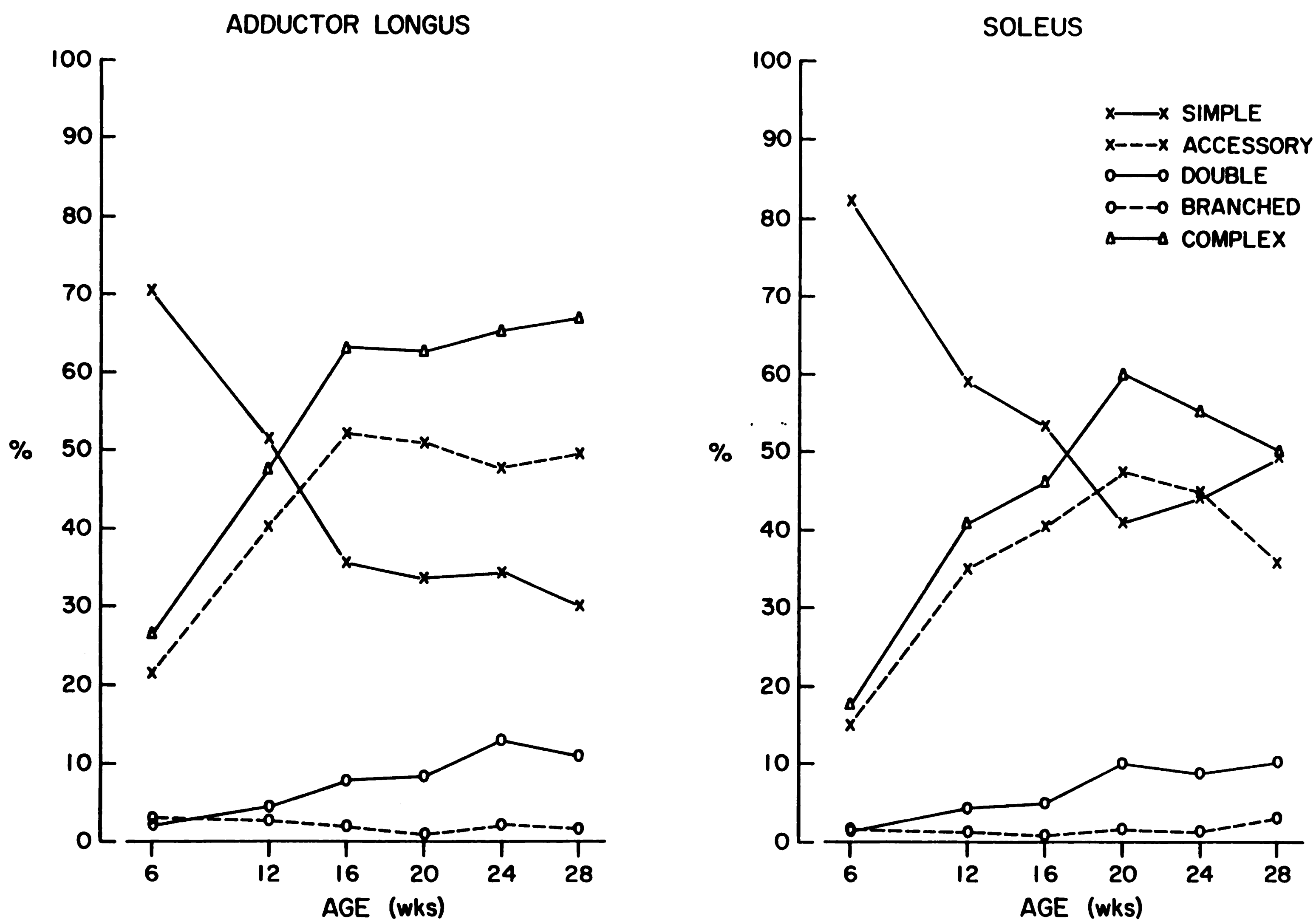
Complexity at the Neuromuscular Junction

The data from this investigation supported and elaborated on that from a previous study (117). Significant differences in terminal ending and endplate structures did exist in selected fast- and slow-twitch muscle preparations. Slow-twitch muscles, post-puberty, demonstrated myoneural junctions which were generally more complex and specifically displayed more accessory and double endings. With maturation it appeared that complexity of endings increased in slow-twitch soleus and adductor longus muscles due again primarily to increases in accessory and double endings

(Figure 4.8). Conversely, terminal arborizations in fast-twitch, post-pubertal muscles appeared to be relatively static (Figure 4.9). The rather low levels of sprouting seen in all muscles in the current investigation may be accounted for by the high percentages of accessory and double endings found. Presumably nodal sprouts had developed to form either accessory or double endings as suggested by Barker and Ip (6) and Tuffery (116). The exceptionally small amount of preterminal and ultraterminal sprouting seen is also in agreement with the findings of Barker and Ip (6) and may partially explain the low percentage branched endings and, consequently, the low level of collateral innervation seen.

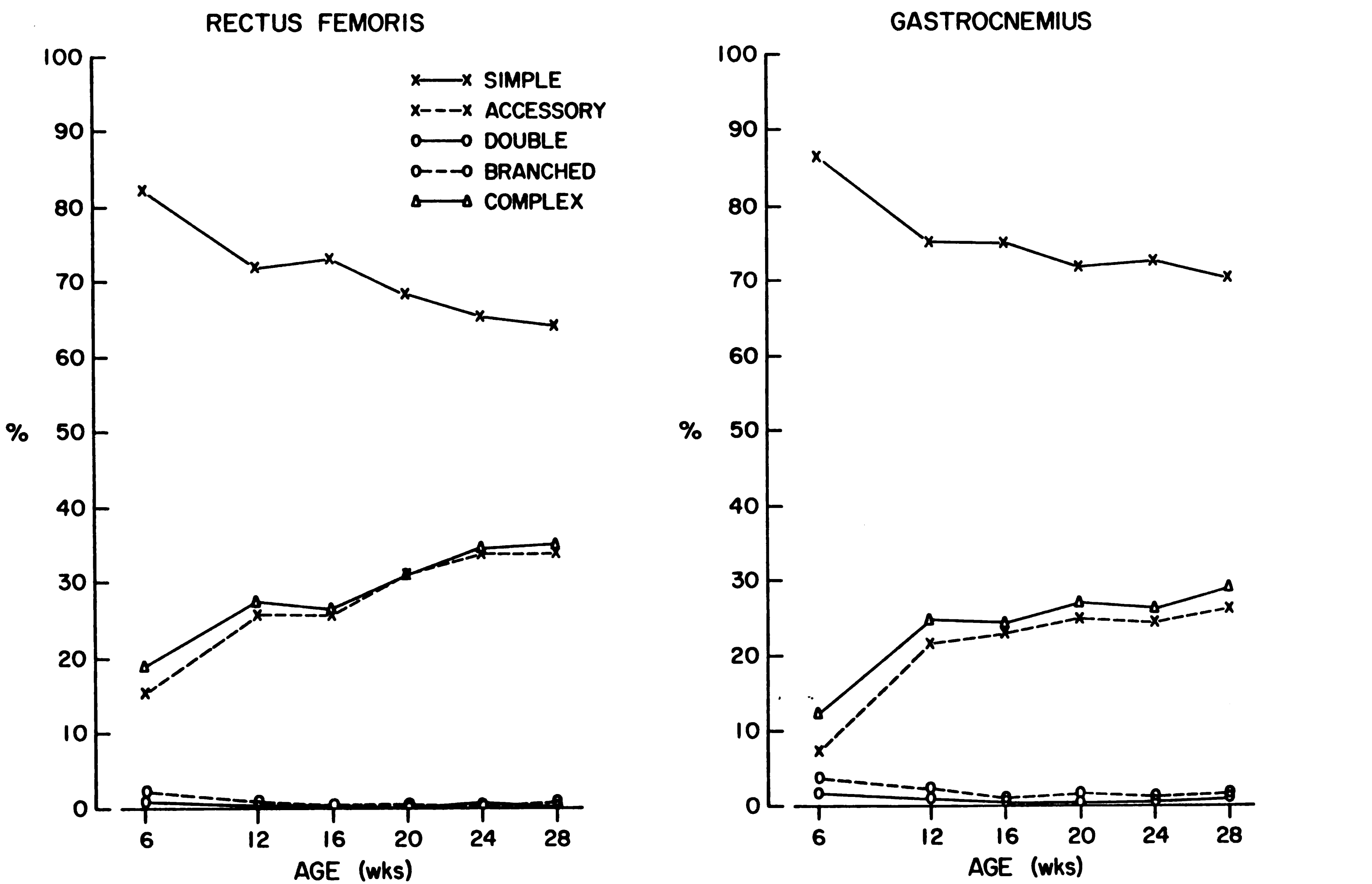
Originally the increases in slow muscle ending complexity were thought to be related, in part, to increased workload in these primarily postural muscles attributable to animal weight gain. This is unlikely as no general pattern of differences in complexity or ending categories was seen when comparing endurance-exercised animals with the significantly heavier control animals. Further, a preliminary study comparing male and female rats of similar ages, but of different weights indicated that there were no weight-related changes present. (Tweedle and Stephens, unpublished).

The increased number of axonal terminals seen in slow-twitch muscles, be they into one or more endplates on a given fiber, may be a compensatory mechanism to ensure impulse transmission at a smaller and less well developed junction. Presumably each branch would conduct a separate impulse into the endplate region (116) thereby activating a greater relative percentage of endplate area per contact. Similarly such increased impulse conduction through additional branches might effectively



CHANGES IN TERMINAL NERVE ENDINGS IN SPECIFIC MUSCLES

Figure 4.8. Changes in terminal nerve endings in specific muscles--adductor longus and soleus.



CHANGES IN TERMINAL NERVE ENDINGS IN SPECIFIC MUSCLES

Figure 4.9. Changes in terminal nerve endings in specific muscles--rectus femoris and gastrocnemius.

compensate for the decreased transmitter release per unit surface area associated with the ageing process. Alternatively the ending/endplate elaboration seen in slow-twitch muscles and with maturation may possibly reflect a system designed to maintain the trophic activity of the motoneuron (116) by providing alternate channels for axoplasmic flow into the expanded endplate area.

Exercise Effects on the Neuromuscular Junctions

Forced endurance exercise, which presumably produced increased activity levels preferentially in FOG and FG fibers without concomitant muscle fiber hypertrophy (86), produced no consistent pattern of changes in terminal arborization morphology. Similar results have been noted in rats allowed voluntary activity in running wheel cages for 10 weeks (Stephens and Tweedle, unpublished). These results suggest that increased activity levels of an endurance nature which do not alter muscle fiber size or speed (Appendix C) cannot alone produce changes in neuromuscular junction morphology.

Tenotomy experiments which result in decreased percentages of double endings (117) as well as causing both atrophy and, in slow-twitch soleus muscle, a shift to a faster contracting muscle (119) further suggest the importance of muscle fiber size or speed of contraction in relation to terminal arborization structure.

CHAPTER V

SUMMARY, CONCLUSIONS, AND RECOMMENDATIONS

Summary

The purpose of the present investigation was to determine the effects of maturation and a chronic endurance-exercise program on the morphology of the terminal motor ending and endplate in both fast-twitch (gastrocnemius and rectus femoris) and slow-twitch (adductor longus and soleus) muscle from normal, male Sprague-Dawley rats.

One hundred animals were brought into the laboratory in two shipments and randomly assigned to one of two treatment groups: control (CON) or endurance running (END). CON animals were sacrificed at 6, 12, 16, 20, 24, or 28 weeks of age. END animals were subjected to a progressive, endurance-running program for 4, 8, 12, or 16 weeks and were sacrificed at 16, 20, 24, or 28 weeks of age respectively.

Determination of fiber type composition for each muscle taken was made on frozen sections using myosin adenosine triphosphatase (ATPase 9.4) (39), and succinic dehydrogenase (SDH) (5).

Muscles taken from the left hindlimb were fixed in 10% formal-saline with 0.5% DMSO for 6 hours, rinsed in distilled water for 16 hours, and then frozen. A minimum of six serial longitudinal sections 60 to 70 micra thick were stained using the combined cholinesterase-Bielchowsky method of Gwyn and Heardman (62) for motor endplate/terminal axon morphology.

Specific determination of morphological characteristics of the terminal arborizations was made using the categorization system and technique developed by Tweedle and Stephens (117). Both age-related changes and exercise-induced modifications were noted. Comparative statistical analyses were done using the Mann-Whitney U-test (111).

Generalized results of the morphological changes have been summarized in Table 5.1.

Conclusions

The results of this investigation have led to the following conclusions:

1. Significant differences exist between the neuromuscular axonal terminals of post-pubertal fast-twitch and slow-twitch muscles. Primarily slow-twitch muscles (adductor longus and soleus) exhibit increased percentages of accessory, double, multiple, and complex endings as well as decreased percentages of simple endings when compared with predominantly fast-twitch muscles (gastrocnemius and rectus femoris).

2. Between 6 (pre-pubertal) and 12 (post-pubertal) weeks of age, significant synaptic rearrangement occurs in all muscles as demonstrated by increased ending complexity. After 12 weeks, increased neuromuscular junction complexity appears to be characteristic of slow-twitch muscles.

Table 5.1. Summary of Major Changes in Terminal Motor Endings

Endplate Category	Fiber Type Related	Age Related	Exercise Related
Accessory	1. Slow-twitch muscle had significantly higher percentages after 12 weeks of age.	1. All muscles had increased percentages up to 12 weeks, thereafter only slow-twitch muscle appeared to increase before plateauing between 16 and 20 weeks.	1. There was a decrease in number of accessories for exercised slow-twitch muscle at 8 weeks.
Double	1. Significant differences existed between fiber types at all ages after 6 weeks.	1. Adductor longus muscle increased percentages up to 24 weeks, while the soleus increased to 20 weeks. 2. No changes in fast-twitch muscle were seen -- percentage double endings was always below 2.0%.	1. Minimal exercise effects.
Branched	1. No trend was readily visible.	1. For all muscles percentages were generally below 2.5% and always below 4.0%. 2. Initial decreases for gastrocnemius and rectus femoris were seen up to 16 weeks, and for the adductor longus up to 20 weeks.	1. Minimal exercise effects.
Simple	1. Slow-twitch muscle demonstrated less simple endings at all points in time except at 6 weeks when the soleus and rectus femoris were similar.	1. A decrease in simplicity occurred for all muscles between 6 and 12 weeks. 2. Slow-twitch muscles continued to decrease before levelling off between 16 and 20 weeks.	1. Increased percentages of simple endings were seen only in slow-twitch muscles and only after 8 weeks of exercise.
Sprouts and Multiple	1. Adductor longus had higher percentages of sprouting than the rectus femoris. 2. Gastrocnemius generally showed more sprouting than the soleus (significant at 6 and 20 weeks). 3. Multiples were seen only in slow-twitch muscle.	1. Usually less than 1.5% sprouting was seen (adductor longus at 6 weeks was the exception) and less than 3.0% multiples were observed.	1. Minimal exercise effects.
Complex	1. Slow-twitch muscle was significantly more complex than fast-twitch muscle at all ages except at 6 weeks when the soleus and rectus femoris were equal.	1. Increased complexity was visible in all muscles examined between 6 and 12 weeks. 2. Slow-twitch muscle complexity continued to rise until plateauing at 16 weeks in the adductor longus and 20 weeks in the soleus.	1. Decreased complexity was visible in the slow-twitch muscles after 8 weeks of exercise.

3. Exhaustive endurance exercise appears to have little effect on the modification of motor nerve endings. Apparently normal maturational nerve ending changes are not due to alterations in neuromuscular activity of this type.

Recommendations

1. A follow-up study should be conducted to assertain the effects of advanced age on terminal motor ending morphology and plasticity.

2. Exercise programs specifically designed to overload the fast-twitch glycolytic and fast-twitch oxidative glycolytic muscle fibers should be implemented with an identical animal population.

3. Cross-innervation and reinnervation investigations should be conducted using the same ending/endplate categorization technique.

4. Attempts should be made to refine the current cholinesterase-Bielchowsky nerve stain such that identification of individual muscle fibers is more easily made.

5. Physiological and ultrastructural studies should be carried out in conjunction with future investigations.

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APPENDICES

APPENDIX A

TRAINING PROGRAM

APPENDIX A

Table A-1. Modified Sixteen-Week Endurance-Training Program for Postpubertal and Adult Male Rats in Controlled-Running Wheels

Wk.	Day of Wk.	Day of Tr.	Acc- eler- ation Time (sec)	Work Time (min: sec)	Rest Time (sec)	Repe- ti- tions per Bout	No. of Bouts	Time Bet- ween Bouts (min)	Shock (ma)	Run Speed (m/ min)	Total Time of Prog. (min: sec)	Total Exp. Revo- lu- tions TER	Total Work Time (sec) TWT
0	4=T	-2	3.0	40:00	10	1	1	5.0	0.0	27	40:00	---	---
	5=F	-1	3.0	40:00	10	1	1	5.0	0.0	27	40:00	---	---
1	1=M	1	3.0	00:10	10	40	3	5.0	1.2	27	49:30	450	1200
	2=T	2	3.0	00:10	10	40	3	5.0	1.2	27	49:30	450	1200
	3=W	3	3.0	00:10	10	40	3	5.0	1.2	27	49:30	450	1200
	4=T	4	2.5	00:20	10	30	2	5.0	1.2	27	34:40	450	1200
	5=F	5	2.5	00:30	15	20	2	5.0	1.2	27	34:30	450	1200
2	1=M	6	2.0	00:40	20	15	2	5.0	1.2	36	34:20	600	1200
	2=T	7	2.0	00:50	25	12	2	5.0	1.2	36	34:10	600	1200
	3=W	8	1.5	01:00	30	10	2	5.0	1.2	36	34:00	600	1200
	4=T	9	1.5	02:30	60	4	2	5.0	1.2	36	31:00	600	1200
	5=F	10	1.0	02:30	60	4	2	5.0	1.2	36	31:00	600	1200
3	1=M	11	1.0	02:30	60	4	2	5.0	1.2	36	31:00	600	1200
	2=T	12	1.0	05:00	0	1	5	2.5	1.2	36	35:00	750	1500
	3=W	13	1.0	05:00	0	1	5	2.5	1.2	36	35:00	750	1500
	4=T	14	1.0	05:00	0	1	5	2.5	1.2	36	35:00	750	1500
	5=F	15	1.0	05:00	0	1	5	2.5	1.2	36	35:00	750	1500
4	1=M	16	1.0	05:00	0	1	5	2.5	1.2	36	35:00	750	1800
	2=T	17	1.0	07:30	0	1	4	2.5	1.0	36	37:30	900	1800
	3=W	18	1.0	07:30	0	1	4	2.5	1.0	36	37:30	900	1800
	4=T	19	1.0	07:30	0	1	4	2.5	1.0	36	37:30	900	1800
	5=F	20	1.0	07:30	0	1	4	2.5	1.0	36	37:30	900	1800
5	1=M	21	1.0	07:30	0	1	4	2.5	1.0	36	37:30	900	1800
	2=T	22	1.0	07:30	0	1	5	2.5	1.0	36	47:30	1125	2250
	3=W	23	1.0	07:30	0	1	5	2.5	1.0	36	47:30	1125	2250
	4=T	24	1.0	07:30	0	1	5	2.5	1.0	36	47:30	1125	2250
	5=F	25	1.0	07:30	0	1	5	2.5	1.0	36	47:30	1125	2250
6	1=M	26	1.0	07:30	0	1	5	2.5	1.0	36	47:30	1125	2250
	2=T	27	1.0	10:00	0	1	4	2.5	1.0	36	47:30	1200	2400
	3=W	28	1.0	10:00	0	1	4	2.5	1.0	36	47:30	1200	2400
	4=T	29	1.0	10:00	0	1	4	2.5	1.0	36	47:30	1200	2400
	5=F	30	1.0	10:00	0	1	4	2.5	1.0	36	47:30	1200	2400
7	1=M	31	1.0	10:00	0	1	4	2.5	1.0	36	47:30	1200	2400
	2=T	32	1.0	10:00	0	1	5	2.5	1.0	36	60:00	1500	3000
	3=W	33	1.0	10:00	0	1	5	2.5	1.0	36	60:00	1500	3000
	4=T	34	1.0	10:00	0	1	5	2.5	1.0	36	60:00	1500	3000
	5=F	35	1.0	10:00	0	1	5	2.5	1.0	36	60:00	1500	3000
8	1=M	36	1.0	10:00	0	1	5	2.5	1.0	36	60:00	1500	3000
	2=T	37	1.0	12:30	0	1	4	2.5	1.0	36	57:30	1500	3000
	3=W	38	1.0	12:30	0	1	4	2.5	1.0	36	57:30	1500	3000
	4=T	39	1.0	12:30	0	1	4	2.5	1.0	36	57:30	1500	3000
	5=F	40	1.0	12:30	0	1	4	2.5	1.0	36	57:30	1500	3000

Table A-1. (continued)

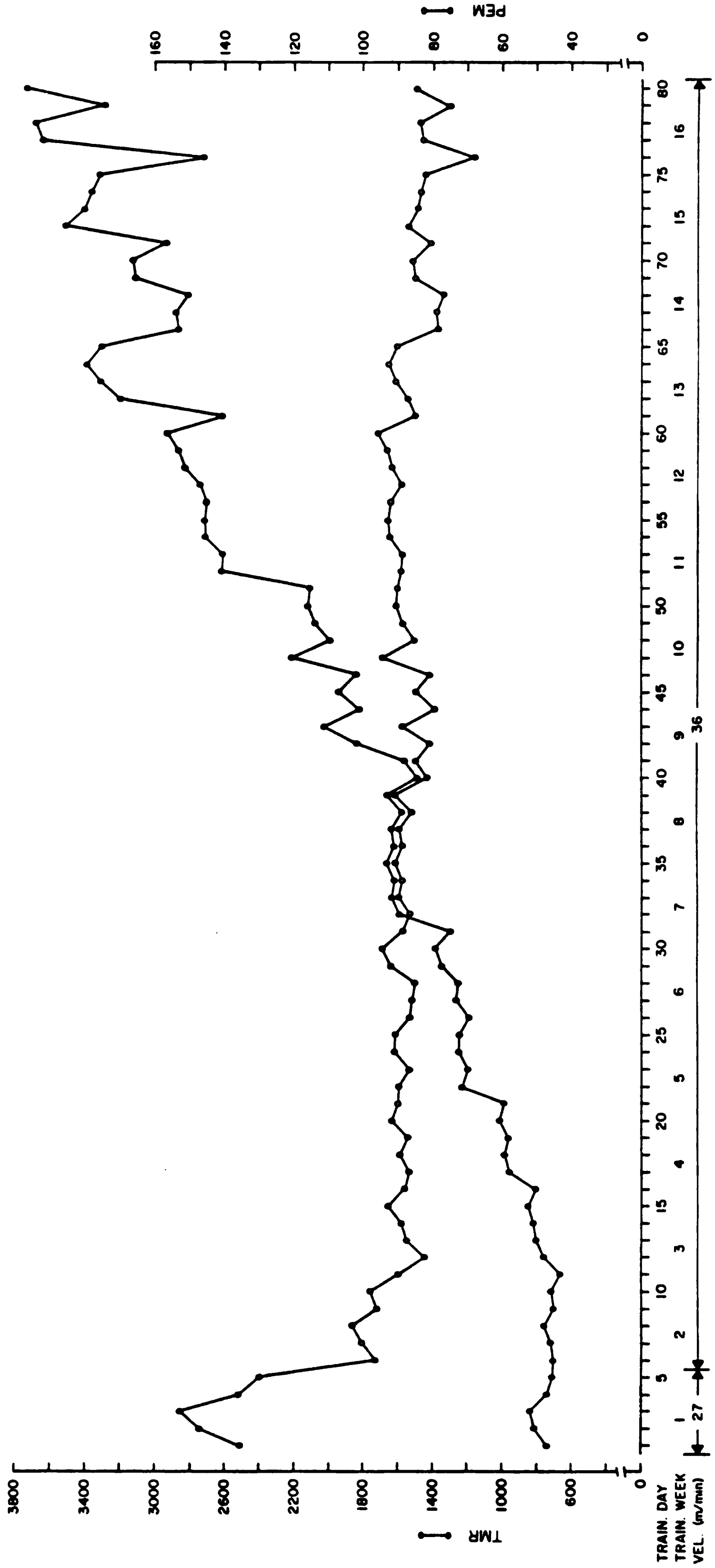
Wk.	Day of Wk.	Day of Tr.	Acceleration Time (sec)	Work Time (min: sec)	Rest Time (sec)	Repetitions per Bout	No. of Bouts	Time Between Bouts (min)	Shock (ma)	Run Speed (m/ min)	Total Time of Prog. (min: sec)	Total Exp. Revolutions TER	Total Work Time (sec) TWT
9	1=M	41	1.0	12:30	0	1	4	2.5	1.0	36	57:30	1500	3000
	2=T	42	1.0	12:30	0	1	5	2.5	1.0	36	72:30	1875	3750
	3=W	43	1.0	12:30	0	1	5	2.5	1.0	36	72:30	1875	3750
	4=T	44	1.0	12:30	0	1	5	2.5	1.0	36	72:30	1875	3750
	5=F	45	1.0	12:30	0	1	5	2.5	1.0	36	72:30	1875	3750
10	1=M	46	1.0	12:30	0	1	5	2.5	1.0	36	72:30	1375	3750
	2=T	47	1.0	16:00	0	1	4	2.5	1.0	36	74:30	1920	3840
	3=W	48	1.0	16:00	0	1	4	2.5	1.0	36	74:30	1920	3840
	4=T	49	1.0	16:00	0	1	4	2.5	1.0	36	74:30	1920	3840
	5=F	50	1.0	16:00	0	1	4	2.5	1.0	36	74:30	1920	3840
11	1=M	51	1.0	16:00	0	1	4	2.5	1.0	36	74:30	1920	3840
	2=T	52	1.0	16:00	0	1	5	2.5	1.0	36	90:00	2400	4800
	3=W	53	1.0	16:00	0	1	5	2.5	1.0	36	90:00	2400	4800
	4=T	54	1.0	16:00	0	1	5	2.5	1.0	36	90:00	2400	4800
	5=F	55	1.0	16:00	0	1	5	2.5	1.0	36	90:00	2400	4800
12	1=M	56	1.0	16:00	0	1	5	2.5	1.0	36	90:00	2400	4800
	2=T	57	1.0	21:00	0	1	4	2.5	1.0	36	91:30	2520	5040
	3=W	58	1.0	21:00	0	1	4	2.5	1.0	36	91:30	2520	5040
	4=T	59	1.0	21:00	0	1	4	2.5	1.0	36	91:30	2520	5040
	5=F	60	1.0	21:00	0	1	4	2.5	1.0	36	91:30	2520	5040
13	1=M	61	1.0	21:00	0	1	4	2.5	1.0	36	91:30	2520	5040
	2=T	62	1.0	20:00	0	1	5	2.5	1.0	36	110:00	3000	6000
	3=W	63	1.0	20:00	0	1	5	2.5	1.0	36	110:00	3000	6000
	4=T	64	1.0	20:00	0	1	5	2.5	1.0	36	110:00	3000	6000
	5=F	65	1.0	20:00	0	1	5	2.5	1.0	36	110:00	3000	6000
14	1=M	66	1.0	20:00	0	1	5	2.5	1.0	36	110:00	3000	6000
	2=T	67	1.0	25:00	0	1	4	2.5	1.0	36	107:30	3000	6000
	3=W	68	1.0	25:00	0	1	4	2.5	1.0	36	107:30	3000	6000
	4=T	69	1.0	25:00	0	1	4	2.5	1.0	36	107:30	3000	6000
	5=F	70	1.0	25:00	0	1	4	2.5	1.0	36	107:30	3000	6000
15	1=M	71	1.0	25:00	0	1	4	2.5	1.0	36	107:30	3000	6000
	2=T	72	1.0	27:30	0	1	4	2.5	1.0	36	117:30	3300	6600
	3=W	73	1.0	27:30	0	1	4	2.5	1.0	36	117:30	3300	6600
	4=T	74	1.0	27:30	0	1	4	2.5	1.0	36	117:30	3300	6600
	5=F	75	1.0	27:30	0	1	4	2.5	1.0	36	117:30	3300	6600
16	1=M	76	1.0	27:30	0	1	4	2.5	1.0	36	117:30	3300	6600
	2=T	77	1.0	30:00	0	1	4	2.5	1.0	36	127:30	3600	7200
	3=W	78	1.0	30:00	0	1	4	2.5	1.0	36	127:30	3600	7200
	4=T	79	1.0	30:00	0	1	4	2.5	1.0	36	127:30	3600	7200
	5=F	80	1.0	30:00	0	1	4	2.5	1.0	36	127:30	3600	7200

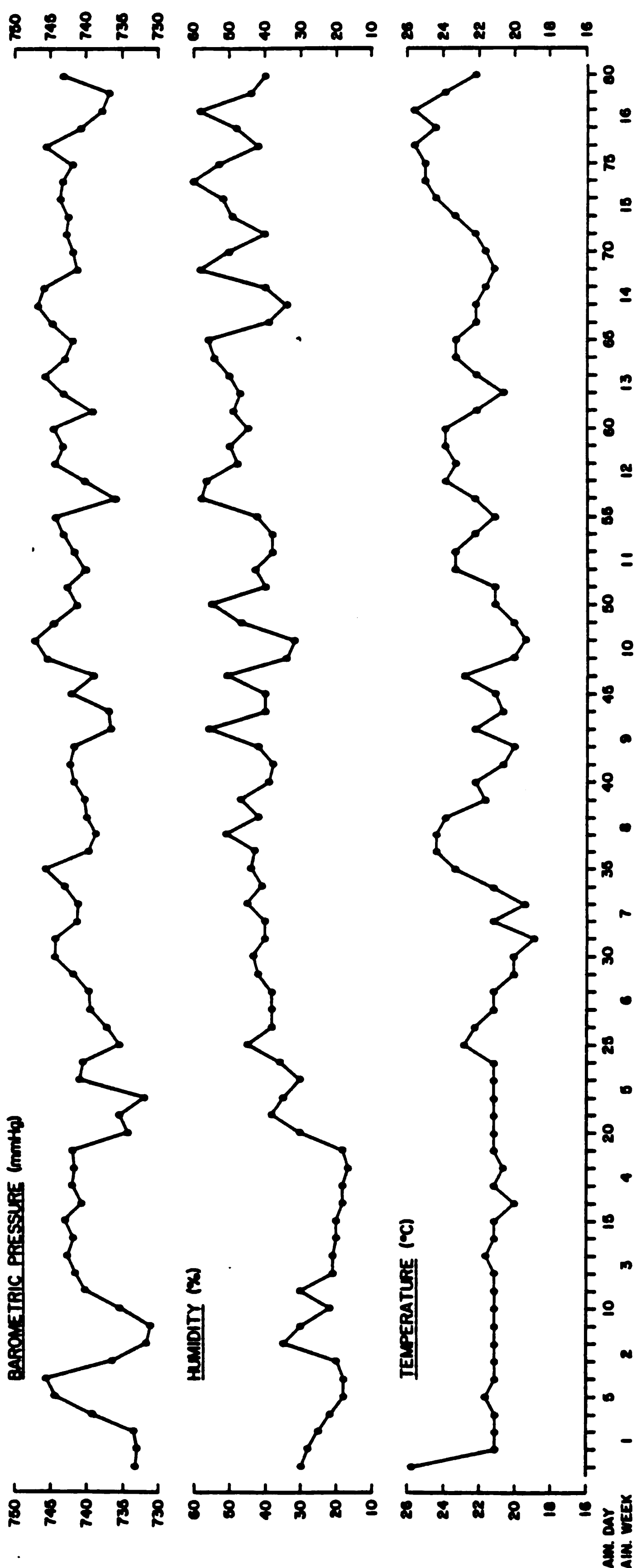
This training program is a modified version of a standard program designed using male rats of the Sprague-Dawley strain (100,107).

All animals should be exposed to a minimum of one week of voluntary running in a wheel prior to the start of the program. Failure to provide this adjustment period will impose a double learning situation on the animals and will seriously impair the effectiveness of the training program.

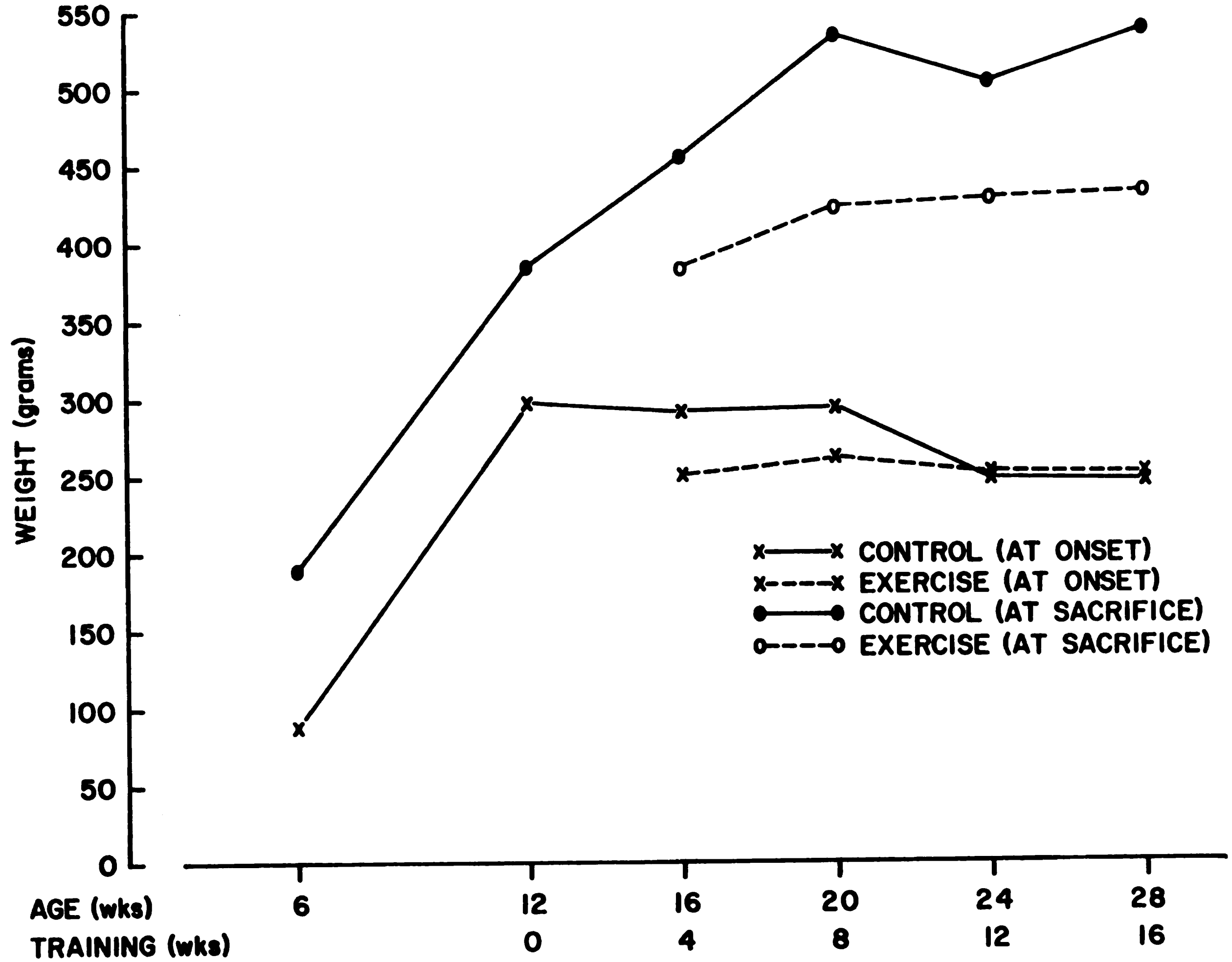
APPENDIX B

TRAINING, TREATMENT ENVIRONMENT, AND BODY WEIGHT VALUES


TMR
PEM
TRAIN. DAY
TRAIN. WEEK
VEL. (m/min)
27
36



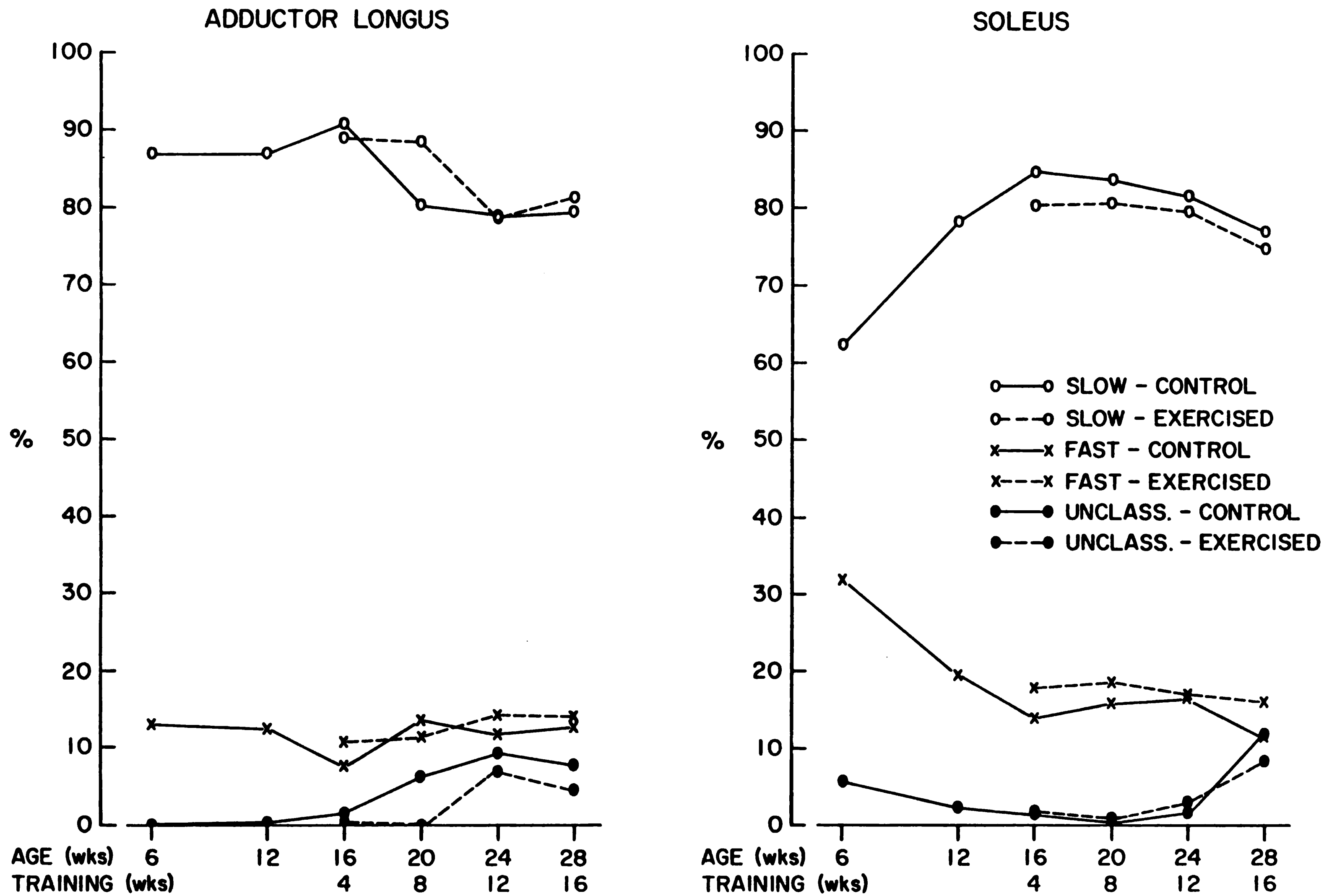
Mean Daily Temperature, Percent Humidity, and Barometric Pressure



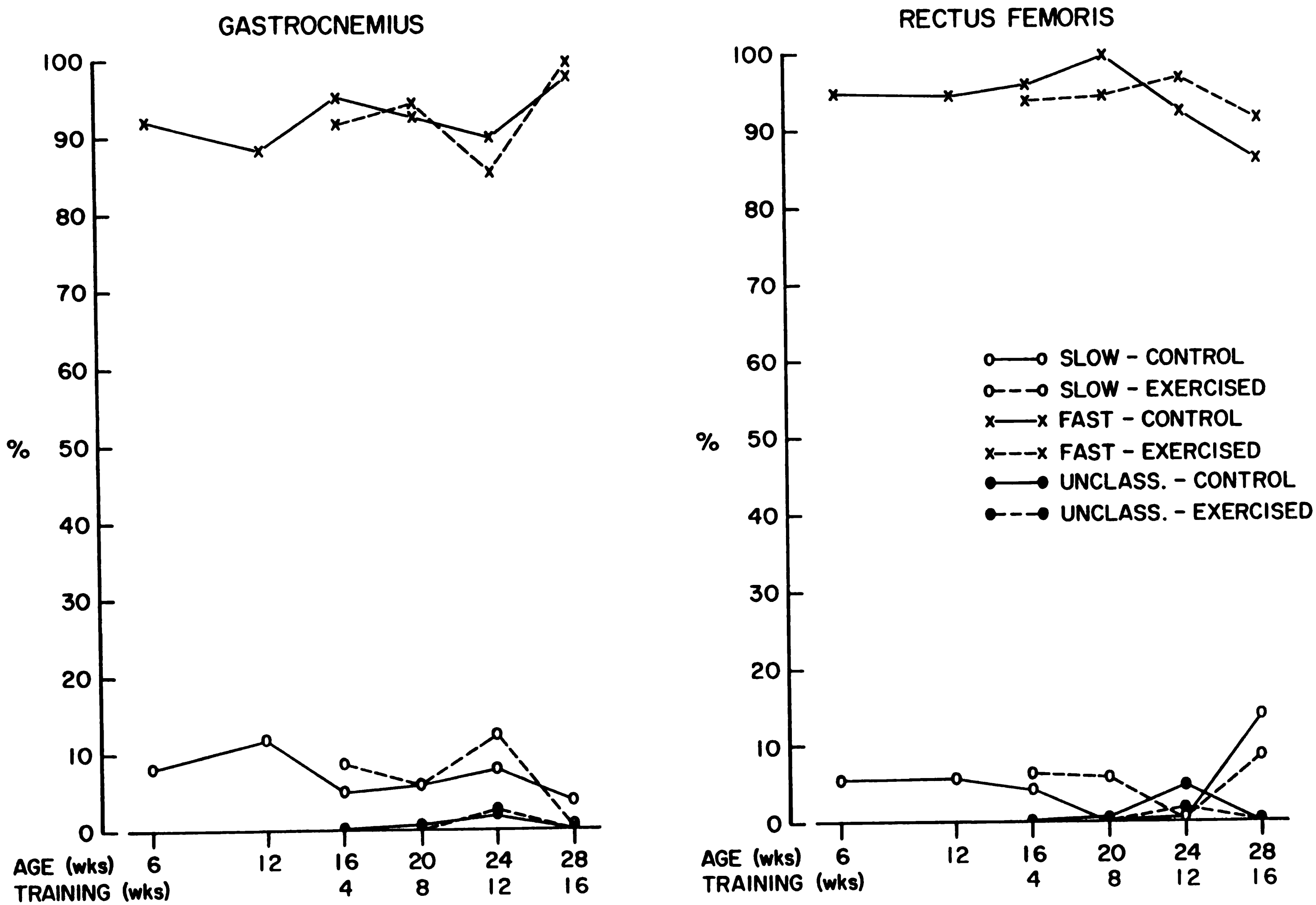
MEAN BODY WEIGHTS AT ONSET OF TREATMENT AND AT SACRIFICE

APPENDIX C

MUSCLE FIBER TYPE DATA



MUSCLE FIBER TYPES IN NORMAL AND ENDURANCE - EXERCISED RATS



MUSCLE FIBER TYPES IN NORMAL AND ENDURANCE - EXERCISED RATS