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THE INFLUENCE OF AXONAL TRANSPORT ON FACIAL MUSCULATURE IN THE GROWING RAT

by

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Abstract

by

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The objectives of the present study were to examine the separate role of axonal transport compared to that of nerve impulse transmission on the development of facial musculature in the growing rat and its secondary influence on the growth of underlying skeletal tissue. In order to differentiate these two neuronal activities, tetrodotoxin (TTX) was used to block impulse conduction selectively without interfering with axonal transport.

The experiment was carried out on 36 male rats initially 14 or 15 days old. The sample was divided into three groups; one control and two experimental. In the control group a constant flow of 0.9% normal saline solution was delivered from an osmotic mini-pump to a special cuff surrounding the facial nerve on the left side. In one experimental group the facial nerve on the left side was cut and the distal stump was extirpated. The purpose was to eliminate both axonal transport and impulse conduction. In the second experimental group 10^{-4} TTX solution was delivered from

an osmotic mini-pump to a cuff surrounding the uncut facial nerve on the left side to achieve prolonged blockage of action potentials without interfering with axonal transport. The total experimental period was 23 days. Animals showing any evidence of nerve damage (non-movement of vibrissae, Wallerian degeneration) were discarded. A succession of three different fluorescent *in vivo* bone markers was administered to measure subsequent bone growth on the periosteal surface of the frontal bone. Biopsies of *M. levator labii superioris* from both operated and unoperated sides were taken prior to sacrifice. Serial cross-sections, 10 µm thick, of the fresh frozen muscle was processed for histochemical demonstration of alkali- and acid-stable myosin ATPase activity. Following sacrifice, 70-90 µm thick serial sagittal bone sections, perpendicular to the fontonasal suture, were prepared.

Interpretations of results were based on intra- and intergroup comparisons regarding (1) diameter measurements of 200 muscle fibers from randomly selected fields, (2) proportion of different muscle fiber types, and (3) histomorphometry of bone apposition at the anterior edge of the frontal bone. Greatest diameter difference was observed in the denervated muscle. Fibers in the experimental sides of the denervated groups were 46% smaller than on contralateral control and saline-treated sides. The TTX-treated muscle fibers showed less severe growth failure but were still 21% smaller in diameter than the control and saline-treated sides. Analysis of variance demonstrated that

muscle fiber diameters following neurectomy were significantly smaller ($p < 0.01$) than the TTX treated, which, in turn, were significantly smaller ($p < 0.01$) than the saline-treated group. These findings indicate that axonal transport as well as impulse activity play an important role in the growth of facial musculature. Histochemical patterns of muscles from both saline-treated and unoperated control sides demonstrated clear differentiation into type IIA and IIB fibers. However, fibers from TTX-treated and denervated muscles remained undifferentiated and did not show segregation into mature histochemical patterns, indicating that impulse activity is a decisive factor in determining changes in the histochemical profile of muscle. Skeletal measurement demonstrated significant differences (p 0.05) in magnitude of appositional bone growth between unoperated and denervated sides and between unoperated and TTX-treated sides. Although the amount of bone accretion on denervated sides was smaller than on TTX-treated sides, analysis of variance failed to show any significant difference between these two groups. These results suggest that appositional growth of bone is more responsive to loss of function of overlying muscular tissue than to changes in muscle fiber size. Based on the composite data, the present study was able to demonstrate the independent role of axonal transport on the growth of the facial musculature and, also, that impaired function and/or size of the facial musculature affect the growth of the underlying osseous tissue.

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DEDICATED

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INTRODUCTION

Most of the functions of the nervous system are known to be associated with impulse conduction and changes in membrane potential at synaptic sites. It has, however, long been recognized that nerves also transmit trophic substances toward the periphery that presumably are necessary for growth and maintenance or regeneration of not only the axon itself but also of the tissues and organs innervated. Although some investigators include impulse conduction as part of trophic modulation, most researchers working in the area of neurotrophism define this phenomenon in the more limited sense of chemical control by axonal transport. For instance, Gutmann (1964) has defined neurotrophic function as long-term maintenance and regulation not mediated by nerve impulse. These functions act homeostatically to maintain and renew the structural and functional capacity of tissues.

Part of the hypothesized mechanism of neurotrophic regulation is that substances synthesized in the nerve cell body are transported toward receptor tissues, as part of the slow, intermediate or fast axonal transport system. In the past, the slow component of axonal transport has been used to refer to proteins transported at rates varying between 1 and 3 mm/day. Identifying groups of polypeptides moving at a given rate within the axon, Lasek and Hoffman (1976) defined two subcomponents, subcomponent a (SCa) and subcomponent b (SCb), of the slow component. SCa is the 1-2 mm/day peak containing microtubules and triplet polypeptides. SCb

is defined as a 3-4 mm/day peak containing tubulin as well as a group of associated proteins. While SCa, which makes up the greater part of the axonal cytoskeleton, is thought to play a major role in the motility of the axon, SCb has been proposed to participate in the outgrowth of regenerating axons (Lasek and Hoffman, 1976). The fast component conveys a large number of different proteins at a rate of 250-400 mm/day. Glycoprotein appears to be a substantial fraction of these proteins (Barker et al., 1975), suggesting that the fast component supplies membranes to maintain the integrity of the axon. Also, fast axonal transport with high dependence on energy metabolism is believed to be related to material essential for impulse conduction and transmission (Ochs, 1972a). In addition, indirect evidence indicates that neurotrophic regulators, which modulate the physiology of target cells such as muscle cells, may be conveyed at relatively rapid rates of transport (Miledi and Slater, 1970). Bi-directional (anterograde as well as retrograde) movement of fast-moving intra-axonal substances has been demonstrated (Lubinska, 1964; Grafstein and Forman, 1980), as has retrograde slow transport (Fink and Grainer, 1980). In both sensory and motor neurons, retrograde transport appears to carry a "signal" substance to the cell body that is thought to control the level of synthesis of axonally transported materials in a negative feedback system. The exact chemical nature of the neurotrophic agents is still unknown and therefore indirect approaches and hypothetical considerations

with regard to experimental investigations are unavoidable. Nevertheless, neurotrophic agents must have the capacity of mediating long-term effects acting relatively independently of impulse activity, and of modulating protein synthesis in the innervated tissue (Gutmann, 1976).

Muscle and muscle fibers manifest differences in structural, contractile, metabolic and membrane characteristics that reflect differences between the motor neurons that innervate them (Buller et al., 1960; Gutmann, 1964; Henneman and Olson, 1965). While the precise mechanism of neural regulation of muscle properties is unknown, experimental data strongly suggest that this regulation is accomplished partly by muscle activity and partly by diffusable, neurohumoral trophic substances carried by axonal transport (Guth et al., 1981). Important changes in muscle tissue following denervation include alterations in membrane properties, contractile properties, ACh sensitivity and receptors, synthesis and degradation of proteins, structures, metabolites and enzymes (reviewed by Gutmann, 1976). Such changes indicate dependency on the neuron, but of course not necessarily on its neurotrophic function.

Denervation atrophy manifests itself as a decrease in the total weight of the muscle, loss of total protein, and reduction of cross-sectional areas of the muscle fibers (Gutmann, 1962). Such atrophy in the limb muscles has been demonstrated to be due to both the disuse caused by paralysis of the muscle, and to the

loss of the neurotrophic substances which are normally secreted by intact motor neurons (Tower, 1935; Gutmann, 1962; Karpati and Engel, 1968; Davis, 1981). In earlier research (Fischbach and Robbins, 1969, 1971; Barnard et al., 1970) comparison of denervation changes with disuse immobilization changes was often used to differentiate neurotrophic influence from impulse activities of the neurons. However, such experimental design does not block nerve impulse activity. The use of tissue culture has provided more reliable evidence of neurotrophic influences on maintenance of muscle structure (Crain and Peterson, 1974). Besides immobilization experiments and tissue culture studies, dissociation techniques have been used in which axonal transport and nerve conduction have been selectively abolished. While antimitotic drugs such as colchicine or vinblastine (Albuquerque et al., 1974) have been used to arrest axonal transport, local anesthetics and tetrodotoxin have been used to interfere with impulse activities. Among these various blocking agents, tetrodotoxin is known to work very selectively on sodium channels without affecting axonal transport (Lavoie et al., 1977). On the other hand, there are possibilities that local anesthetics block axonal transport as well as action potentials (Fink et al., 1972). Colchicine and vinblastine have been used by several investigators (Albuquerque et al., 1972; Hoffmann and Thesleff, 1972; Cangiano, 1973) who have shown denervation-like changes in normally innervated muscles. However, interpretation of such results is complicated

since colchicine or vinblastine can affect muscle fibers by a systemic direct action (Cangiano, 1973; Lømo, 1974) without producing a detectable block of axonal transport (Cangiano and Fried, 1977).

The concept of neurotrophism as a stimulus for oro-facial growth was introduced by Moss (1971,1972) in a speculative way to attempt to explain the regulation of functional matrices. According to Moss, the genetic and epigenetic determinants of skeletal development of the face are not to be found in the bone itself but rather in the functional soft tissue matrices. He hypothesized that all functional matrices are specifically regulated by a trophic process originating in the nervous system. This theory does not, however, rule out the possibility of bone growth and maintenance to be closely influenced by direct neural control of the skeletal tissue itself (Singh et al., 1982).

Although a number of investigators have shown neural influence on facial musculature and skeletal tissue by the use of denervation experiments (Washburn, 1946; Sarnat et al., 1977; Phillips, 1982), strict dissociation experiments have not been used in order to separate neural activities and then to evaluate the possible neurotrophic role in craniofacial growth and development. Thus, the purpose of this study was to elucidate the influence of axonal transport on growth and differentiation of facial musculature and its possible secondary effect on underlying osseous tissues in the growing rat through an in vivo dissociation experiment.

STATEMENT OF THESIS

The objectives of this research project were to test, through a dissociation experiment, the hypotheses (1) that growth and maintenance of the facial muscles, as part of the soft tissue draping of the face, are dependent upon axonal transport, and (2) that this trophic control mechanism secondarily influences the growth and form of underlying osseous tissues.

In an experimental group of young rats, nerve impulse transmission was chemically arrested in the facial nerve without interfering with non-impulse activity. This was accomplished by the use of implanted osmotic mini-pumps which continuously delivered tetrodotoxin (TTX) to the facial nerve. The project also included a second experimental group with neurectomy of the facial nerve, and a control group with mini-pumps delivering saline solution. In order to evaluate bone growth, polychrome fluorescence labeling of bone formation was performed by sequential injections of three *in vivo* bone markers; oxytetracycline, DCAF, and alizarin komplexon.

Interpretation of data was based on muscle histochemistry of *musculus levator labii superioris*, and on fluorescence microscopy of hard tissue sections of nasal and frontal bones with interposed suture. Inter- and intragroup comparisons were made regarding (1) muscle fiber diameter, (2) proportion of muscle fiber types, and (3) histomorphometry of bone apposition at the anterior edge of the frontal bone.

BACKGROUND

Historical Perspective

Although most of the functions of the nervous system are known to be associated with impulse conduction and changes in the membrane potential at synaptic sites, it has long been recognized that nerves also transmit chemical substances toward the periphery that are necessary for growth and maintenance or regeneration of the tissues and organs innervated (Dubner et al., 1978). The hypothesis that the nerve cell body acts as a nutritive center for the axon is evident from the classic observation that the axon degenerated after injury of the nerve cell (Ramon y Cajal, 1928). Later, Rámon y Cajal (1952) pointed out that "perhaps the neuroplasm, situated among the neurofibrils in the cell body and in the axon, serves as a diffusion pathway for a substance or substances still unknown, which have a trophic action and are elaborated by the nucleus". The reality of such axonal transport has been well-documented by a number of investigators (Weiss and Hiscoe, 1948; Lubinska, 1964; Kerkuit, 1968, 1975). Classic work by Weiss and Hiscoe (1948) demonstrated proximodistal flow of axoplasm within the axon. They constricted axons and observed that over a period of weeks axoplasm accumulated proximally to the constriction. Upon release of the constriction, the accumulated axoplasm was observed to move down somato-fugally at a rate of approximately 1 mm/day. These

investigations suggested the presence of substances in the cell body which entered the axon and flowed towards the axon terminals. Kerkuit (1967) placed a ^{32}P label in both the X and XII cranial nerve nuclei and demonstrated peripheral accumulation of the label. Crushing of these nerves prevented passage of the label below the point of mechanical blockage. In this way, the cell body continually supplies the axon with the nutrients necessary for the maintenance of its structural and functional integrity. The axon itself lacks an endogenous protein synthetic machinery other than the rather limited synthetic capacity present in axonal mitochondria (Lasek, 1970). Although glial cells supply axonal proteins as a secondary source, the neuron cell body provides most of the axonal proteins (Lasek et al., 1974). In addition to the trophic function of anterograde transport in maintenance of the axon itself, there is increasing evidence for a transcellular trophic mechanism, whereby some material, passing from the axon to the tissue that it innervates, promotes the survival of that tissue. Transcellular transfer of axonally transported material was first demonstrated by Korr et al. (1967). They placed isotopic labeled compounds in the XII cranial nerve nucleus and later observed their location in the lingual musculature. Recently, selective retrograde transsynaptic transfer of proteins, such as tetanus toxin, has been demonstrated (Schwab et al., 1979). This experiment clearly

suggests the proximal flow of the proteins are responsible for such retrograde trophic influences.

Although some investigators (e.g., Drachman, 1974) include impulse conduction in neurotrophic modulation, most investigators working in the area of neurotrophism define neurotrophism in the more limited sense of chemical control by axonal transport. Thus, Gutmann (1964) defined neurotrophic function as long-term maintenance and regulation not mediated by nerve impulses. These functions act homeostatically to maintain and renew the structure and functional capacity of both parts. A broader definition has been proposed, e.g., Guth (1969) defined neurotrophism as "interaction between nerve and other cells which initiate or control molecular modification in other cells". However, present terminology restricts the definition to axonal transport control.

Rates of Flow, Mechanisms and Substances Involved in Axonal Transport

Since Weiss and Hiscoe (1948) first described "axoplasmic flow" as a slow process of transport related to migration of axoplasm from cell body to periphery, Miani (1960,1962), among others, reported on transport of a considerable rate (fast transport). Other investigators (Karlsson and Sjöstrand, 1971) have reported on the existence of a discrete component of transport with a velocity intermediate between fast and slow transport. Bi-directional (anterograde, retrograde) movement of fast moving intra-axonal particles were also demonstrated by

Lubinska (1964) as has retrograde slow transport (Fink and Gainer, 1980). The velocity of the fast component ranges between 300 mm/day and 450 mm/day in mammalian axon (Ochs, 1972b; Sjöstrand, 1969), and the transport velocity seems to be constant for corresponding nerves within different animals. For example, the maximum rate of axonal transport is about 410 mm/day in mammalian sciatic nerve and is constant in various mammals regardless of the size of the animal and hence the absolute length of the axons (Gross and Kreutzberg, 1978). However, the velocity is not the same in all mammalian nerves; the value in the hypoglossal nerve has been reported to be 300 mm/day (Gurd, 1977; Guth, 1968). The proteins of the slow component are transported at approximately 1-2 mm/day, which corresponds to the rates of axon regeneration. The intermediate components are transported at rates between that of the fast and slow components (Willard et al., 1974; Karlsson and Sjöstrand, 1971). Recently, Lorentz and Willard (1978) reported on five different components of axonal transport by detecting various labeled proteins passing through nerve in the rabbit optic system. Maximum velocities of these are at least 240 mm/day (Group 1), 34-68 mm/day (Group II), 4-8 mm/day (Group III), 2-4 mm/day (Group IV), and 0.7-1.1 mm/day (Group V). Their results have, in general, been accepted by other researchers in the field.

Available data suggest that each component of axonal transport can be related to the movement of identifiable

structures within the axon, and that the structures conveyed in each component are specific to that component, (Droz et al., 1975; Lasek and Hoffman, 1976). This supports the validity of differentiation of axonal transport on the basis of velocity. The material conveyed in the fast axonal transport, such as glycoproteins (Forman et al., 1971), phospholipids (Grafstein et al., 1975), glycosides (Forman and Ledeen, 1972) and neural transmitters, are primarily associated with the membranous cellular structures in the axon. These structures include smooth endoplasmic reticulum, plasma membrane, vesicles of various kinds (including synaptic vesicles), AChE - containing elements, and some mitochondria. In addition to membrane constituents, amino acid, sugar and nucleosides and Ca^{++} were shown to be carried by fast transport (Grafstein and Forman, 1980). Yet, most proteins transported rapidly have not been identified.

Slow transport, on the other hand, involves many cytoplasmic materials including a number of soluble enzymes. Slow transport is also thought to convey the cytoskeletal structures of axons, the microtubules, neurofilaments (Black, 1978), and actin-containing microfilaments. This was first suggested on the basis of electron microscopic radioautographic studies (Droz and Leblond, 1963; Droz et al., 1973) and strongly supported by identification of tubulin, the protein subunit of microtubules in the slow component from a variety of neurons (McEwen et al., 1971; Hoffman and Lasek, 1975). On the basis of recent studies (Black and

Lasek, 1980; Lasek and Hoffmann, 1976), the slow component is subdivided into two subcomponents, designated slow component a (SCa) and slow component b (SCb). SCa is the 1-2 mm/day peak containing microtubules and the triplet polypeptides. SCb is defined as a 3-4 mm/day peak containing tubulin as well as a group of proteins different from the triplet.

Concepts about the molecular mechanisms that drive axonal transport remain hypothetical. The possible mechanisms for fast transport that are currently popular can be classified into three groups (Grafstein and Forman, 1980): (1) a mechanism that involve the related application of small forces to the transported organelles by structures anchored throughout the length of axon ("ratchet mechanisms") (Schmitt, 1968; Ochs, 1974; Kerkuit, 1975); (2) a mechanism in which the transported organelles are swept along by movements of intra-axonal fluid, and (3) mechanisms in which transport takes place within a continuous system of axonal SER (Droz et al., 1973,1975). A peristaltic mechanism was suggested for slow axonal transport by Weiss (1948). Time-lapse films of neurons in vitro were used to show slow rhythmic proximodistal contraction waves along the axon (Weiss et al., 1961). This could not be confirmed by other investigators (Spencer, 1972). Later, an entirely different mechanism of slow axonal transport was proposed (Brady and Lasek, 1979; Black and Lasek, 1980). The findings that myosin may be associated with neurofilaments of the cytoskeletal network to

play a role in motile process and that actin appears to be attached relatively stationary to the inner surface of the axolemma (Chang and Goldman, 1973) led Lasek and Hoffman (1976) to propose a model for the movement of the axonal cytoskeleton based upon actin- myosin interactions. By this mechanism the entire core of microtubules and neurofilaments, all linked together, would be slowly propelled down the axon by pressure entered against the axon membrane.

The functional significance of axonal transport has been reviewed by Ochs (1972b), Gutmann (1976) and Grafstein (1980). In both sensory and motor neurons retrograde transport appears to carry a "signal" substance to the cell body that is thought to control the level of synthesis in a negative feedback system. Whereas SCa, which makes up the greater part of the axonal cytoskeleton, is thought to play a major role in the motility of the axon, SCb participation in the outgrowth of regenerating axons has been proposed (Lasek and Hoffman, 1976). The fast axonal transport with high dependence on energy metabolism is thought to be related to materials essential for impulse conduction and transmission (Ochs, 1972a). Also, materials conveyed from the cell body by fast axonal transport are used to replace axonal proteins, glycoproteins and other materials lost by local breakdown, exocytosis into the synaptic cleft, retrograde migration to the cell body, and synaptic turnover.

Multiple Regulation of Muscle Growth and Function

Different mechanisms, not only neural (impulse, non-impulse) but also non-neural (myogenic, hormonal and humoral), are involved in regulation of the properties of muscle cells. Muscle and muscle fibers have differences in structural, contractile, metabolic and membrane characteristics that reflect differences among the motor neurons that innervate them (Buller et al., 1960; Gutmann, 1964; Henneman and Olson, 1965). However, primary myogenic factors, apparently involving genetic mechanisms, may operate without neural influences. Therefore, myofibers mature in the total absence of innervation, but neural influences are necessary for differentiation, postnatal growth and maintenance. For instance, elaboration of contractile protein (Farmbrough and Rash, 1971), sensitivity to androgens (Hanzlíková and Gutmann, 1974) and to AChE (Fambrough and Hartzell, 1974) are controlled at the myogenic level and are then, at later stages of development, modified by neural influences.

The various changes in muscle following denervation provide evidence of muscle cell dependence on nerve. The changes in membrane properties, ACh sensitivity and receptor, synthesis and degradation of proteins, structure, metabolites, and enzyme related to excitation-contraction coupling, and energy supply and utilization following denervation have been investigated with great interest (reviewed by Gutmann, 1976). These changes are, of course, responsible not only for neurotrophic (non-impulse)

function but also for impulse activities. Melichna and Gutmann (1974) demonstrated changes of histochemical fiber pattern in a denervated muscle by stimulation. Again, Gutmann and co-workers (1975) showed the independence of the changed dynamic properties of denervated muscle on the length of sectioned nerve. If it were dependent on axonal transport, shorter distal fibers would show changes earlier than longer fibers. These experiments suggest that impulse activity is the decisive factor determining changes in the dynamic properties of muscle and fiber histochemical pattern. On the other hand, strong evidence for a neurotrophic mechanism is revealed by analysis of the time of appearance of changes following nerve section made at various distances from the muscle. This is because impulse activity is eliminated, a priori, as a responsible factor. For instance, degeneration of terminal axons (Gutmann et al., 1955), changes in membrane properties (Miledi and Slater, 1970; Tucek, 1975; Luco and Eyzaguirre, 1955), increase of proteolytic activities (Gutmann, 1969), decrease of glycogen synthesis (Gutmann et al., 1975), decrease of resting membrane potential (Albuquerque and Schuh, 1971), appearance of TTX-resistant action potentials (Harris and Thesleff, 1972), and other comparable disturbances appear earlier in the muscle with a shorter nerve stump. These findings pointed to a progressive depletion of trophic substances occurring earlier in a shorter piece of a nerve.

Interpretation of denervation changes is sometimes difficult due to the interaction of neurotrophic and impulse activities of the neurons. Experimental techniques that selectively abolish either action potential conduction or axoplasmic transport serve to distinguish the neurotrophic effects from the effects of impulse conduction and functional demands. This dissociative approach has been particularly effective in the study of neurotrophic relations in muscle (Albuquerque et al., 1974; Gutmann, 1976).

Tissue culture studies have shown that neurotrophic mechanisms are independent of nerve impulse transmission. Muscle atrophy in long-term culture is prevented by explantation into a spinal cord-ganglion culture; recovery and differentiation proceed even after months of isolation of cultured muscle cells (Crain and Peterson, 1974). The addition of sympathetic ganglion chains to a culture medium, supplemented with nerve growth factor (NGF) to enhance outgrowth of the sympathetic neurons, could also retard atrophy in "cord-denervated" muscle fibers and even muscle contraction could be evoked (Crain and Peterson, 1974). Clonal cell lines from neuroblastoma can form neuromuscular contacts with clonal lines of skeletal muscle cells (Harris et al., 1971) after toxin-binding to ACh receptors and after the nerve cells had depleted their stores of ACh and could not be activated by ACh (Patrick et al., 1972).

The importance of nerve presence for newt limb regeneration was reported early (Singer, 1952). The neurotrophic phenomenon has been shown to be associated with non-specific chemical agents, which can also be extracted from the brain, in the regulation of limb regeneration (Singer, 1974).

Although the neurotrophic influence on muscle is usually considered to be mediated by the motor innervation, studies in animals (Pimenidis and Gianelly, 1977) and humans (Bratzlavsky, 1973) show that loss of sensory innervation can alter the proportion of slow to fast muscle fibers.

Even though neural influences on muscle are distinctive, it is important to distinguish the different components affecting (1) the muscle cell directly and (2) the nerve-muscle cell interaction. Most hormones have a regulatory action on some phase of growth, development and maintenance of muscle and may have effects similar to other growth promoting factors (Gutmann, 1976). For example, the androgen-dependent levator ani muscle of the female rat can be maintained after testosterone administration and temporarily after perinatal denervation (Burešová et al., 1972), as the hormone has a relative nerve-independent effect during early stages of development. The myotrophic hormone influence on the target muscle results in increase of weight and muscle fiber size, but not in reversal of muscle fiber patterns which is dependent on innervation (Hanzlíková and Gutmann, 1972). There are few examples of humoral mechanisms

that may affect nerve-muscle cell interactions. Developmental changes in blood flow may be an important factor in differentiation of metabolic muscle fiber types (Hudlická, 1973).

Neurotrophic Regulation of Craniofacial Growth

Since neurotrophic effects have been identified for tissues, organs and limbs, a process for neurotrophic control has been postulated in the development of the face. The attainment of adult oro-facial form results from the integration of a number of growth processes and regulatory mechanisms. Moss and Young (1960) introduced the functional matrix theory, which is based on the work of van der Klaauw (1946,1948,1951,1952) to partially explain the control of facial growth processes. According to Moss, the genetic and epigenetic determinants of skeletal development are in the functional soft tissue matrix, not in the bone itself. Bone tissue is essentially passive, and its response is secondary in a chain of control factors regulating contiguous structures. Solow and Kreiborg (1977) have also suggested that growth and the ultimate size and shape of facial osseous structures are closely associated with the facial soft tissue draping. It is important to understand that the functional matrix principle describes what happens during growth, but does not account for how it happens (Enlow, 1975). To describe the growth control process more comprehensively, two problems in relation to the functional matrix principle await exploration (Moss, 1971). The first is the nature of the functional matrix-skeletal tissue interface,

that is, how stimuli are transmitted across this interface. The second is the nature of the complex of specific factors that regulate and control the growth of functional matrices themselves. The concept of neurotrophism in relation to oro-facial growth stimulus was proposed by Moss (1971,1972) in a speculative way to attempt to explain the developmental regulation of the functional matrices. He hypothesized that all functional matrices, capsular and periosteal, are specifically regulated by a trophic process originating in the nervous system. It was theorized that neurotrophic processes provide for long- term interactions between neurons and their innervated tissues by homeostatically regulating the morphological, compositional and functional integrity of those tissues. The neurotrophic processes thus would initiate and control molecular modification in these same tissues (Moss, 1972). This theory, however, would not rule out the possibility that bone growth and maintenance can be closely influenced by direct neural control of the bone tissue itself. Recently, neural regulation of osteogenesis was investigated by sensory and autonomic denervation of growing bone and monitored by ^{3}H-proline autoradiography of osteoblastic activity (Singh et al., 1982). They concluded that sensory as well as sympathetic denervation resulted in a reduction of osteoblastic activity and resultant formation of bone matrix. However, as the authors pointed out, it was not possible to dissociate impulse transmission from axonal transport. In order

to evaluate the role of neurotrophic control in craniofacial growth, strict dissociation experiments are necessary. This has also been pointed out by Dubner et al. (1978).

Neural influence on craniofacial growth has been investigated in denervation studies by many investigators (Washburn, 1946; Rogers, 1955; Sarnat et al., 1977; Gardner et al., 1980; Phillips, 1982). However, these experiments have primarily focused on evaluation of function/form alteration following denervation of motor nerves innervating facial or masticatory musculature and did not evaluate the roles of nerve impulse activity and neurotrophic effects as separate factors. Thus, Washburn (1946) observed deformation and asymmetry of the maxilla and other facial bones following resection of peripheral facial nerves in the neonatal rat and rabbit. Rogers (1955) reported on changes in form of the temporomandibular joint (TMJ) in monkeys as a result of unilateral section of the third division of the trigeminal nerve. However, no skeletal changes were observed by Sarnat et al. (1977) in adult monkeys utilizing similar procedures. These inconsistencies of experimental results might be species related and the initial age of selected nerves on functional matrices. Recent denervation experiments by means of unilateral electrolytic lesions on trigeminal motor nuclei in growing guinea pigs (Gardner et al., 1980) and in an adolescent monkey (Phillips et al., 1982) demonstrated distinct and clinically noticeable facial asymmetries. Phillips et al.

(1982) described alterations in craniofacial morphology including atrophy of the muscles of mastication, mandibular dental asymmetry, changes in TMJ structure and remodeling changes in the orbit and various facial sutures.

Frank and Karli (1962) demonstrated atrophy of the masticatory muscles, localized pathologic changes in the oral muscosa and within the salivary glands, and thinning of the fibrocartilage covering the condyle in the rat 8 to 10 days following the placement of electrolytic lesions in the region of the sensory and motor nuclei of the trigeminal nerve. They concluded that lack of trophic function of the trigeminal nerve was a contributing factor underlying these changes. However, their experimental design made neurotrophic influence possible through the peripheral nerve due to the centrally located lesions in relation to the cell bodies of the sensory neurons. Behrents (1975) placed unilateral electrolytic lesions at three different sites relative to the trigeminal ganglion; anterior (peripheral) to the ganglion, in the ganglion (Gasserian) and posterior (central) to the ganglion. He noted only small differences in a few skeletal roentgenographic measurements and concluded that neurotrophic effects on growth of the face are correspondingly small. However, Dubner et al. (1978) felt that this result cannot be attributed exclusively to a neurotrophic deficit, since impulse conduction was also abolished, a condition which was evidenced by the author's statement that "some of the animals

reacted to the facial anesthesia produced by the operation by constantly scratching the ipsilateral face".

Although speculation dealing with possible relationships between neurotrophism and facial development is intriguing, little evidence has been presented to date to support or rebute this conjecture. Strict dissociation experiments have thus far not previously been used.

MATERIAL AND METHODS

Sample

The experiments were carried out on 36 male Sprague-Dawley® rats[1]. They were 14 or 15 days old at the beginning of the experimental period.

The sample was divided into three different groups: Group I (Control) comprised five animals. A constant flow of 0.9% normal saline solution was delivered from an osmotic mini-pump to a cuff (described below) surrounding the facial nerve on the left side (Fig. 4,5). The contralateral side was left untouched. Group II (Experimental 1: Neurectomy) comprised 11 animals. The facial nerve on the left side was cut. Distal clumps of nerve were removed. The contralateral side was untouched. Group III (Experimental 2: tetrodotoxin, TTX) comprised 20 animals. To achieve prolonged blockage of action potential, 10^{-4} M tetrodotoxin[2] was delivered from an osmotic mini-pump to a cuff surrounding the facial nerve on the left side. The contralateral side was untouched. The rat was selected as the experimental animal for the following reasons:

(1) It is a hardy animal, that can be expected to withstand the physical and psychological stress of the experiment.

(2) It is possible to separate pups from mother before normal weaning age, provided they are hand-reared for a couple

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[1]Zivic-Miller Laboratories Inc., Allison Park, PA

[2]Sigma Chemical Company, St. Louis, Missouri

days. Following separation, weight gain is similar to naturally weaned pups.

(3) Data available in the literature regarding normal muscle fiber growth and histochemistry of different muscle fiber types for this species.

The initial animal weights ranged from 30g to 55 g. The experiments were begun when the animals were 14 or 15 days of age, and the following condition existed:

(1) Postnatal growth in diameter of type II muscle fibers in the rat is greatest during the second and third weeks (Curless and Nelson, 1976).

(2) Differentiation and maturation of type II muscle fiber in rat is very critical during the third and fourth postnatal weeks (Brooke et al., 1971).

(3) Following a surgical procedure it is possible to force wean the young animals at 14 days post-partum (own observation).

(4) Animals are large enough to receive an osmotic mini-pump implanted subcutaneously in the hypodermis back.

Experimental Period

The total experimental period comprised 23 days and included three phases. The initial phase was three days. On the first experimental day, when the animals were 14 or 15 days old, a cuff and a connecting tube were implanted around the facial nerve on the left side in Groups I, II and III. Animals were observed for the

following two days to detect possible nerve damage from the cuff. Lack of vibrissa movement was used as an indicator of possible nerve damage. The animals that did not show normal movement were discarded.

The second phase began on the fourth experimental day and continued for 14 days following. On day four, animals in Groups I and III underwent a second surgical procedure for implantation of an osmotic pump containing saline solution for Group I and TTX solution for Group III. Only animals that showed normal movement of vibrissae (Fig. 1) were used for Groups I and III. During this second phase, chemicals were delivered to the facial nerve on the left side at a constant rate, and paralysis of facial musculature developed (Fig. 2). Animals in Group II underwent neurectomy of the left facial nerve on the fourth experimental day in this phase. The facial nerve on the left side was cut and distal stumps were removed.

The third phase comprised the last 7 days of the experimental period. During this period, TTX or saline solution gradually stopped flowing and Group III animals were thus allowed to recover from paralysis of the left facial musculature. Regaining of vibrissae movement was used as indicator of recovery. If this did not occur the animal was discarded.

Figure 1 -- Animal from saline-treated group. Vibrissae indicates normal alignment and bilateral symmetry. No nasal deviation.

C: untreated control side

X: saline-treated side

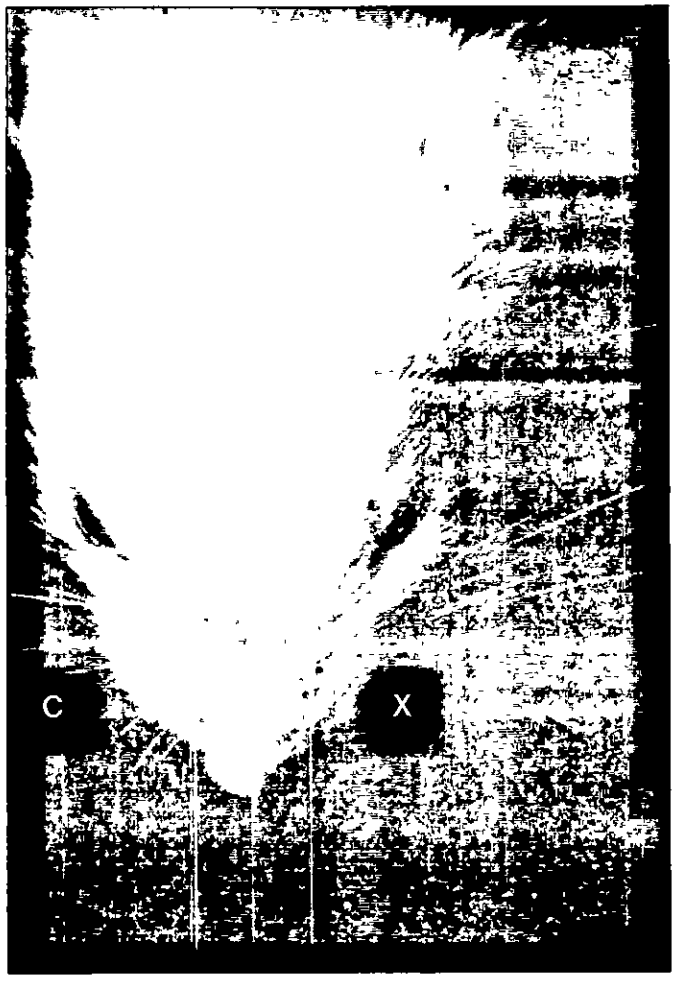

C
X
10
20
30
40
50
60
70

Figure 2 -- Animal from TTX-treated group. Vibrissae shows bilateral asymmetry with those of the treated side pointing posteriorly. Note nasal deviation towards untreated sides.

C: untreated control side

X: TTX-treated side

Similar conditions were observed in the animals from the Neurectomy group.


C
X

Design of Device for Chemical Administration

A device was designed for chronic application of chemical at a constant flow rate to localized areas, thus, minimizing systemic effects.

The device was composed of three components (Fig. 3):

(1) Osmotic mini-pump (Alzet, Model 2002)[1]. This pump consists of a cylindrical reservoir for the fluid, surrounded by a layer of an osmotic driving agent which, in turn, is encapsulated by a semipermeable membrane. When the filled pump is placed in an aqueous environment, the osmotic agent imbibes water at a rate that is controlled by the semipermeable membrane. The imbibed water generates hydrostatic pressure on the flexible lining of the reservoir, gradually compressing it, producing a constant flow of its content through the delivery portal. Experiments in vivo and in vitro have demonstrated that this pump requires about 6 hours to achieve a stable output but will then pump steadily for two weeks (Theeuwes and Yum, 1976). With a 200 µl reservoir the Model 2002 Alzet osmotic pump will give a flow rate of 0.5 µl/hr (at 37°C) during these two weeks.

The complete pump system with a flow modulator in place has the following dimensions: length, 3.0 cm; diameter, 0.7 cm; weight (empty), 1.1 g; total volume, 1 ml. The pump was implanted in a subcutaneous pocket on the back of the animal.

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[1]Alza Corporation, Palo Alto, California, U.S.A.

(2) Cuff enclosing the nerve. The cuff was used to keep the opening of the tubing from the pump in the vicinity of the nerve and to prevent the chemicals from diffusing into contiguous tissues too rapidly. Silastic® Medical-Grade Tubing[1] (0.058 in. inner diameter, 0.077 in outer diameter) was cut into 3 mm lengths. A joint was made between the cuff and the tubing from the pump (connecting tube, see below). Silastic® 382 Medical-Grade Elastomer[1] was used as adhesive. After joint formation, a complete slit was made along the cuff in order to slide it over the nerve. The polymerized elastomer was penetrated with a sharp instrument in the joint area between connecting tube and cuff to allow chemicals to freely flow from osmotic pump to the inside of the cuff. The length of the cuff was adjusted during implantation to prevent mechanical irritation of the nerve. The cuff fits quite loosely over the nerve and is very flexible so as to avoid mechanical irritiation.

(3) Connecting tube. A connecting tube led from the osmotic pump to the cuff. Silastic® Medical Grade Tubing (0.30 in. ID, 0.65 in. OD)[1] was cut into 3 cm lengths and connected to the middle portion of the cuff. The cuff-connecting tube unit was implanted during the initial surgery. The distal end of the connecting tube was connected to the flow modulator of the osmotic pump during the second surgical procedure. Between the first and

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[1]Dow Corning Corporation, Medical Products, Midland, Michigan

second surgical procedures this end of the connecting tube was sealed with Silastic 382 Medical Grade Elastomer to prevent it from being filled with tissue fluid.

Determination of TTX Concentration

TTX solutions of various concentrations, 10^{-3} M, 10^{-4} M, 10^{-5} M, 10^{-6} M, and 10^{-7}M were prepared in advance. Following general anesthesia, the facial nerve was exposed in 15 day old pilot rats. A piece of parafilm was placed under the facial nerve to prevent the diffusion of TTX solution and electrical current during testing procedure. Before the application of the TTX solution to the nerve, the untreated nerve was tested with an electrical stimulus to obtain a reference measurement. The untreated nerve responded at 50 volts with a 10 msec. single pulse. Responsiveness was easily detected by vibrissa movement resulting from facial muscle twitch. Then, a piece of cotton was soaked with a TTX solution and applied to the exposed nerve for 2-3 min. The nerve was lifted carefully from the underlying tissue with an electrode and stimulated with 50 volts of electricity with a 10 msec single pulse. When the nerve was treated with 10^{-3} M and 10^{-4} M TTX solution, muscle twitch was not evoked, even at 70 volts. To determine the lowest concentration with effectiveness *in vivo*, 10^{-3} M, 10^{-4}M and 10^{-5} M TTX solutions were filled in osmotic mini-pumps which were implanted in pilot animals. The 10^{-5} M TTX solution was not effective in blocking the action potential. On the other hand, the 10^{-3} M TTX solution showed

toxic side effects in the young animals. Thus, the 10^{-4} M TTX solution was used in the main experiment.

Anesthesia

An adequate level of anesthesia was obtained using an intramuscular injection of Ketamine HCl[1]. Two mg/kg body weight was used for animals under 100 g body weight and ten mg/kg body weight for animals over 100 g. To prepare animals for muscle biopsy, an intramuscular injection of 5 mg/kg body weight of ketamine HCl was followed by an intraperitoneal injection of 10-12 mg/kg body weight of Sodium Pentobarbital[2].

Surgical Procedures for Implantation of Drug-Releasing Device

(1) Implantation of cuff and connecting tube (Fig. 4). At 14 or 15 days of age the experimental animals were separated from their foster mothers and weighed. Under anesthesia and antiseptic conditions, a 6 or 7 mm oblique incision was made in regio colli lateralis extending anteriorly over the mandibular ramus. After reflecting overlying soft tissues including musculature, the facial nerve was exposed and freed carefully under a dissecting microscope (X 20). The section of the nerve to receive the cuff is immediately proximal to the point where the nerve divides into a buccalis and a marginal mandibular branch.

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[1]Ketaset, Veterinary Products, Bristol Laboratories, Syracuse, New York.

[2]Diabutal, Diamond Laboratories, Inc., Des Moines, Iowa

Figure 3 -- Device for chemical administration.

P: osmotic mini-pump (Model 2002,Alzet)

T: connecting tube

C: cuff

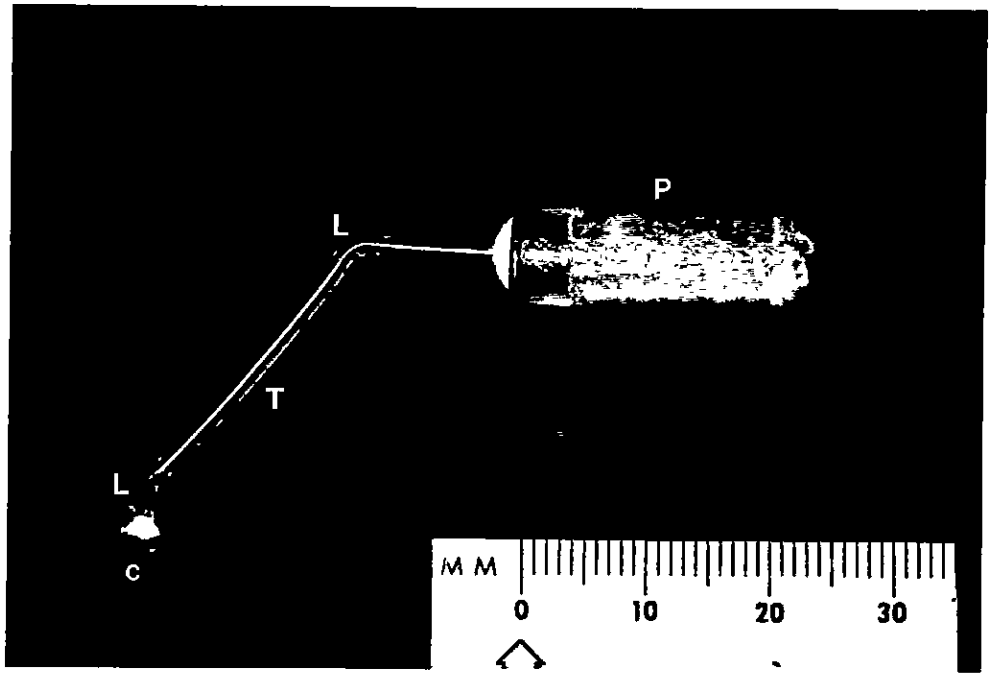
L: ligature wire for permanent angulation of connecting tube.

Figure 4 -- First surgical procedure. Implantation of cuff-connecting tube unit.

N: Facial nerve

C: Cuff

T: Connecting tube


P
L
T
L
C
M M
0
10
20
30


T
C
N

A subcutaneous pocket was made in regio colli posterior extending latero-anteriorly towards the first incision, thus creating a pathway for the connecting tube. After sterilization in Providone-iodine solution (Betadine®) and washed with normal saline solution, the connecting tube was inserted into the prepared pocket. A steel ligature was tied around the tube to form a permanent bend in the tube thus keeping the cuff parallel to the facial nerve to prevent tearing of the nerve. The freed nerve was lifted and placed into the cuff through the open longitudinal slit.

Silastic® 382 Medical Grade Elastomer was used to seal the slit in the cuff. Reflected tissues were approximated and the incision was sutured with 4-0 silk. Animals were kept on the heating pad until they had completely recovered from anesthesia. After surgery the animals were kept separately from their foster mothers to avoid cannibalism. They were fed with liquid baby formula[1].

(2) Implantation of osmotic mini-pump (Fig. 5). This surgical procedure was only performed on animals that showed normal movement of vibrissae two days after implantation of the cuff and connecting tube. Group I and III experimental animals underwent this surgical procedure. A cutaneous incision was made in the regio colli posterior at the level of the cervical vertebrae. From this open-

[1]Enfamil, Mead Johnson and Company, Evansville, Indiana.

ing a subcutaneous pocket was formed caudally with a pair of hemostatic forceps to prepare space for the osmotic mini-pump. The filled osmotic mini-pump (with normal saline solution or 10^{-4} M TTX solution) was inserted into the subcutaneous pocket. Experimental solutions were also injected into the connecting tube and cuff with a microsyringe[1] from the distal end of the connecting tube which was then connected to the metal tip of the flow modulator and secured with 4-0 silk suture. The incision was closed with 4-0 silk suture.

(3) Neurectomy. In order not to introduce an additional variable, the animals to be subjected to neurectomy (Group II) also received a nerve cuff and a connecting tube on experimental day 1, following similar procedures as for Groups I and III. The neurectomy itself was performed on experimental day 4. A new incision was made in the left regio colli lateralis which extended anteriorly over the mandibular ramus. The cuff and tube were removed and the facial nerve was cut proximal to the point where it branches into a buccalis and a marginal mandibular branch. The distal parts (the two branches) were extirpated.

Vital Dyes for Bone Labeling

In order to facilitate histomorphometric measurement of sutural growth, sequential polychrome fluorescence labeling of bone formation was performed. A series of three different in vivo bone

[1]Hamilton Company, Reno, Nevada

markers was administrated in each rat at three different times during the experimental period. At the beginning of the experiment, 30 mg/kg body weight oxytetracycline[1] was administered intramuscularly. On the tenth day, 15 mg/kg body weight of DCAF (2,4 Bis)-N,N'DI(carboxyl-methyl)[2] mixed in 2% $NaHCO_3$ solution was administered intraperitoneally. Thirty mg/kg body weight of Alizarin Complexon[3] mixed in 2% $NaHCO_3$ solution was administered intraperitoneally 24 hours prior to sacrifice.

Muscle Histochemistry

1) Procedure for muscle biopsy (Fig. 6). - Right and left M. levator labii superioris from all experimental animals were prepared for histochemical study. Gastrocnemius and Soleus were also removed and used as reference material since they have known histochemical profiles (Brooke and Kaiser, 1970).

The following steps were employed for the preparation of muscle tissues prior to the histochemical procedures (Dubowitz and Brooke, 1973).

1. Biopsies, 6-8 mm long, were excised from the middle portion of the M. levator labii superioris under general

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[1]Terramycin oxtetracycline of 250 mg/ml, Pfizer Laboratories, Division of Pfizer, Inc. N.Y., N.Y.

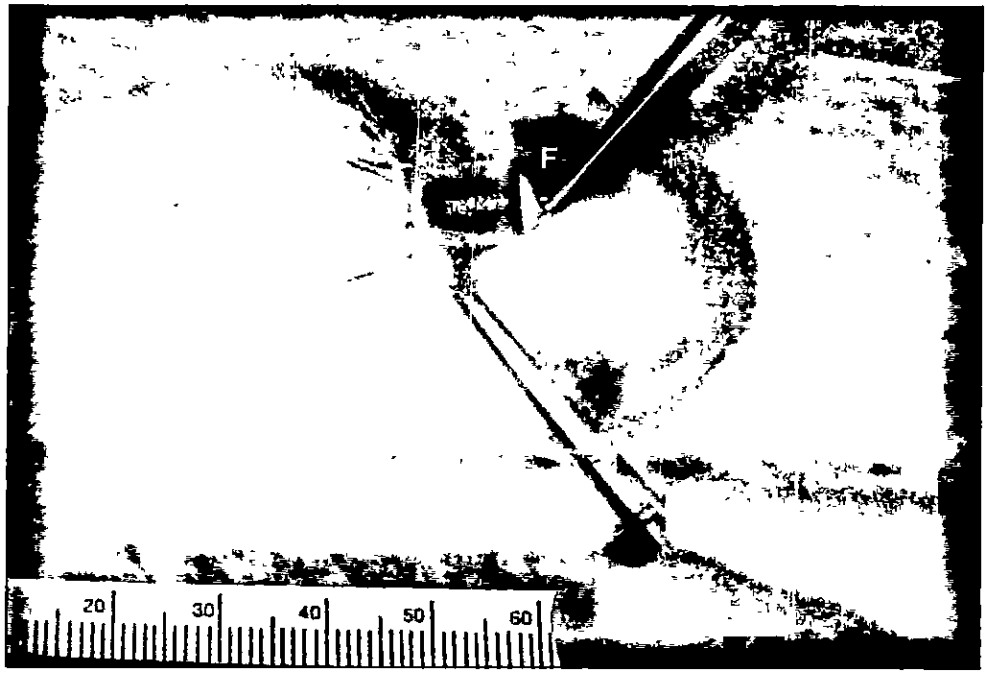
[2]Aminomethyl Fluorescein, Life Sciences. Group of ICN phamaceuticals, Inc., Cleveland Ohio

[3]Eastman Kodak Company, Rochester, N.Y.

Figure 5 -- Second surgical procedure. Implantation of osmotic mini-pump. The connecting tube was attached to the flow modulator on the pump.

F: flow modulator

Figure 6 -- Prior to sacrifice, surgical removal of M. levator labii superioris for ATPase histochemistry.


F
20
30
40
50
60



anesthesia but without local anesthesia (Fig. 6). Also, biopsies from gastrocnemius and soleus were taken.

2. The muscle biopsies, fixed to a piece of cork and vertically oriented with the help of Cryoform[1], were frozen by plunging them into isopentane cooled to about -160°C with liquid nitrogen. This was done immediately after removal from the surgical site .

3. Serial cross-sections, 10 μm thick, were cut in a cryostat[1].

4. The sections were positioned on warm, clean glass slides and air-dried at room temperature for an hour prior to chemical processing as described below.

2) Methods for Calcium-Activated ATPase. The procedure (Chayen et al., 1969) involves the following steps:

1. Preincubation at pH 4.6: half of the sections from each muscle were preincubated at pH 4.6 for 5 minutes. Reaction medium: 0.2 M sodium acetate (27.2 g/1), 45 ml; 0.2 M acetic acid (11.55 ml gs. 1L), 55 ml.

2. Blotting of the preincubation solution remaining on the slides with clean tissue paper.

3. Incubation at pH 9.4: preincubated sections and the rest of untreated sections were incubated in freshly prepared

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[1]International Cryostat, IEC, Model CTO, Needham Heights, MA.

reaction medium at 37°C for 30 minutes. Reaction medium: 0.1 M sodium barbituate solution (2.06 g/100 ml), 10 ml; ATP, 76 mg; 2.4 dinitrophenol, 30 mg; 2% calcium-chloride solution (10.5 g, $CaCl_2.2H_2O$/100 ml), 5 ml; distilled water 35 ml.

4. Wash in three changes of 1% calcium chloride for two minutes in each bath.
5. Rinse in running distilled water for 2 minutes.
6. Transfer to 1% cobalt nitrate solution for 2 minutes then wash in two changes of the same solution for 2 minutes.
7. Wash thoroughly in running distilled water to remove adsorbed cobalt for 4 minutes.
8. Place in dilute ammonium sulfide (0.5% v/v) for one minute.
9. Wash in distilled water for two minutes.
10. Mount the sections with A-Q aqueous mounting medium[1].

ATPase activity is shown by a brown-black precipitate of colbalt sulphide.

Hard Tissue Preparation

After the animals were sacrificed with an overdose of pentobarbital sodium by cardiac injection, the skulls were placed in 50% alcohol for 6 hours, then transferred to 70% (6 hrs), 95% (6

[1]Hopkins and Williams, Chadwellhealth, Essex, England

hrs) and finally to absolute alcohol. They were kept in absolute alcohol until ready for embedding in Bio-Plastic[1]. Skulls were cleaned of all soft tissue by boiling in water and then sectioned along the coronal suture. The anterior parts of the skulls were embedded in Bio-Plastic according to the following steps:

1. Cleaned specimens dehydrated in 50%,70%,95%, and absolute alcohol (30 minutes in each solution).
2. Specimens placed in xylene for 15 minutes.
3. Fresh xylene for another 15 minutes.
4. Specimens immersed in polymerizing Bio-Plastic® (mixture of resin and hardner).
5. Let cured in Bio-Plastic® 60°C for 48 hours.

Serial sagittal sections perpendicular to fronto-nasal suture were cut at 70-90 μm on a high speed precision bone slicer specially designed for thin hard tissue sections.

Histologic Preparation of Nerve Tissue

To examine for possible Wallerian degeneration in the nerve tissues which received the cuffs, nerve tissues were removed prior to sacrifice. They were immediately fixed in Bouin's fluid (unsaturated aqueous picric acid, 750 ml; 40% formaldehyde, 150 ml; acetic acid, 100 ml) until ready for staining. The tissues were embedded in paraffin according to the routine procedures. Serial

[1]Ward's, Rochester, N.Y.

cross-sections at 8 μm tickness were prepared and impregnated with silver protein by Bodian's method.

The following procedures were employed for nerve staining:

1. Deparaffinize sections with xylene and hydrate them.
2. Impregnate in 1% silver protein solution in a 36°C oven for about 20 hours.
3. Wash 6 times with double distilled H_2O.
4. Place in reducing fluid for 10 minutes. Reducing fluid: Hydroquinone, 1g; sodium sulfite, 5 g; double distilled H_2O, 100 ml; potassium metaborate, 0.5 ml.
5. Wash 6 times with double distilled H_2O.
6. Place in gold chloride for 3 minutes. Gold chloride: gold chloride, 1 g; double distilled H_2O, 10 ml; acetic acid, 4 drops.
7. Wash 3 times with distilled H_2O.
8. Place in 1% oxalic acid until sections become grey (about 3 minutes).
9. Wash 3 times with distilled H_2O.
10. Place in 5% sodium thiosulfate for 5 minutes.
11. Wash in running tap water for 10 minutes.
12. Rinse with double distilled H_2O.
13. Dehydrate up to xylene.
14. Mount.

Morphometric Procedure

A. Muscle Measurement

A "typical area" in terms of histochemical appearance was selected under low magnification (X31.25) and photomicrographs were taken at a magnification of X200 using Kodak technical pan film 2415 (ESTAR-AH Base)[1]. Prints were made at a final magnification of X442.

The outlines of the total number of individual muscle fibers that fell within a 10 cm by 10 cm area were traced on tracing paper. When a fiber touched the borderline of the area, its entire outline was completed, even though part of the fiber may have extended beyond the outline square. A unit square area included about 200 muscle fibers. Muscle fiber diameters were determined by measuring the maximum distance across the lesser aspect of the muscle fiber (Brooke, 1973). This procedure was used to avoid artifacts introduced by oblique sectioning of the muscle which will give an artifically large measurement. Measurements were taken with a Helios precision needle-jaw dial caliper and read to 0.1 mm.

B. Bone Measurement

Undecalcified serial sections from both experimental and control animals were obtained for the purpose of evaluating possible influences of facial musculature on the growth of underlying

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[1]TP 135-36, Eastman Kodak Company, Rochester, N.Y.

bone. Three sections from each side were selected for measurement. They were matched, according to distance from the midline, with their counterparts of the contralateral side. Under fluorescence illumination, the three in vivo bone markers could clearly be identified. Oxytetracycline, which was given on experimental day 1 stains the ossification front yellow, DCAF, which was given on day 10, stains the front light green, and Alizarin Komplexon, given on day 22, stained the front red. Appositional bone growth from day 10 to the end of the experiment was measured (Fig. 7). Thus, measurements were taken between the DCAF and the Alizarin Komplexon lines. Three measurements were made along the superoposterior edge of the frontonasal suture on the frontal bone. The perpendicular distance from the rostral surface (stained with Alizarin Komplexon) to the point where the two DCAF lines converge, indicating the location where sutural and surface appositional growth meet, was measured (Fig. 7). The measurements were made with an eyepiece micrometer and taken to the nearest 5 μm.

Figure 7 -- Histomorphometry of skeletal tissue.

O: oxytetracycline

C: DCAF

A: alizarine komplexon

S: front nasal suture

B: Frontal bone

D: distance measured indicator of appositional growth from day 10 to end of the experiment.



RESULTS

Weight Gain

Individual differences in animal weight gain occurred during the experiment, but analysis of variance did not show differences between the groups to be significant at the 5% level of confidence (Table I).

Gross Examination of Skull

As soon as the animals were sacrificed, the skulls were cleaned of the overlying skin and other soft tissues to determine the presence of any gross skeletal deviations or facial asymmetries. None of the animals subjected to either denervation (Neurectomy group), saline treatment (Control group) or blockage of nerve impulse (TTX-group) showed detectable topographic changes by direct visual examination.

Morphometry of Muscle Fiber

Intra- and intergroup comparisons of muscle fiber diameters were made to evaluate influence of impulse activity and axonal transport on muscle fiber growth. Mean diameter of 200 muscle fibers from each muscle in randomly selected fields (Table II,III) were statistically analyzed according to the following steps: (1) The contralateral control sides from each group were compared by analysis of variance (Table IV). No significant difference was found among the three groups. (2) Paired t-tests were performed

TABLE I

Weight Data; Initial Weight, Final Weight and Weight Gains

Animal		Initial Weight (Grams)	Mean (Grams)	S.D. (Grams)	Final Weight (Grams)	Mean (Grams)	S.D. (Grams)	Weight Gain (Grams)	Mean (Grams)	S.D. (Grams)
Group I (Saline)	1	37			215			178		
	2	43	38	3.4641	220	220	5.0000	177	181	6.0828
	3	37			225			188		
Group II (Neur-ectomy)	1	37			198			161		
	2	40			210			170		
	3	40			226			186		
	4	40			185			148		
	5	36	41.2	6.9250	190	215.4	25.1272	154	174.2	19.4239
	6	36			212			176		
	7	35			202			167		
	8	51			226			175		
	9	55			270			215		
	10	45			235			190		
Group III (TTX)	1	38			222			184		
	2	38			210			172		
	3	38			201			163		
	4	36			220			184		
	5	35	35.1	2.8038	222	193.8	27.4535	187	158.7	25.2290
	6	34			179			145		
	7	32			152			120		
	8	35			180			145		
	9	30			158			128		

TABLE II

Data on Muscle Fiber Diameter

		Control Side			Experimental Side		
Group	#	Xi (mm)	$\bar{x}$ (mm)	α (mm)	Xi (mm)	$\bar{x}$ (mm)	α (mm)
Saline	1	9.0228			8.7425		
	2	9.8233	8.8636	1.0484	8.4156	8.5506	0.1707
	3	7.7447			8.4937		
	1	6.9039			5.0322		
	2	7.0691			3.4092		
	3	7.8598			5.6028		
	4	8.6232			4.0013		
Neurec.	5	10.0062	8.1565	1.1511	4.6625	4.5303	0.9379
	6	7.7819			6.2815		
	7	8.5511			4.7428		
	8	7.7261			3.6671		
	9	10.0547			3.4862		
	10	6.9893			4.4176		
	1	8.1083			5.7353		
	2	7.0991			7.2963		
TTX	3	7.6852			6.5926		
	4	8.3897			5.2047		
	5	8.7017	7.9928	0.5949	7.2617	6.2923	0.8034
	6	7.7535			6.6565		
	7	8.5924			5.3283		
	8	7.4251			5.7437		
	9	7.1800			6.8118		

TABLE III

Mean: Muscle Fiber Diameters in μm

	Control Side		Experimental side	
Group	Mean (μm)	α	Mean (μm)	α
Saline	20.0	2.4	19.3	0.4
Neurec.	18.5	2.6	10.2	2.1
TTX	18.1	1.3	14.2	1.8

TABLE IV

ANOVA for Muscle Fiber Diameters: Unoperated Sides of the Three Groups

Source	Degree of Freedom	Sum of Squares	Mean Square	F Value	PR > F	R-Square	CV
Model	2	1.7221	0.8611	0.96	0.3989	0.092206	11.5399
Error	19	16.9548	0.8924				
Corrected Total	21	18.6769					

for intra-group comparison (right and left sides within each group). These results are presented in in Table V. The control group revealed no difference between contralateral control and saline-treated sides. However, both experimental groups showed significantly ($p < 0.01$) decreased fiber diameters on the experimental side (neurectomy or TTX- treatment) in comparison with the contralateral, control side. (3) The operated sides of the two experimental groups, TTX-treated and neurectomy, respectively, were compared by t-test (Table VI). Results showed that muscle fibers were significantly smaller ($p < 0.01$) following neurectomy compared to those of the TTX-treated sides.

Muscle Histochemistry

Skeletal muscle fibers are not homogeneous with regard to their chemical and physiological characteristics. On the basis of certain features three basic fiber types may be differentiated: type I, type II A, and type II B. Type I fibers, with a predominantly oxidative metabolic profile, are slowly contracting and are relatively resistant to fatigue. On the other hand, type II B fibers, with a mainly anaerobic or glycolytic profile, have a shorter contraction time and show rapid fatigue. Type II A fibers are endowed with both aerobic and anaerobic metabolic mechanisms; they contract rapidly but at the same time are fatigue resistant. Table VII summarizes the histochemical profile of each fiber type according to myosin ATPase activity.

TABLE V

Paired-Comparison t-Tres: Unoperated and Operated Sides Within Each of the Three Groups

Group	#	Control (mm)	Experi (mm)	Diff. (mm)	Mean (mm)	STD. Error of Mean	T	PR > /T/
Saline	1	9.0228	8.7425	-0.2803				
	2	9.8233	8.4156	-1.4077	-0.3130	0.6228	-0.50	0.6651
	3	7.7447	8.4937	0.7490				
NEUREC.	1	6.9039	5.0322	-1.8717				
	2	7.0691	3.4092	-3.6599				
	3	7.8598	5.6028	-2.2570				
	4	8.6232	4.0013	-4.6219				
	5	10.0062	4.6625	-5.3437	-3.6262	0.5099	-7.11	0.0001
	6	7.7819	6.2815	-1.5004				
	7	8.5511	4.7428	-3.8083				
	8	7.7261	3.6671	-4.0590				
	9	10.0547	3.4862	-6.5685				
	10	6.9893	4.4176	-2.5717				
TTX	1	8.1083	5.7353	-2.3730				
	2	7.0991	7.2963	0.1972				
	3	7.6852	6.5926	-1.0926				
	4	8.3897	5.2047	-3.1850				
	5	8.7017	7.2616	-1.4400	-1.7005	0.4123	-4.12	0.0033
	6	7.7535	6.6565	-1.0970				
	7	8.4924	5.3283	-3.2641				
	8	8.5251	5.7437	-2.6814				
	9	7.1800	6.8118	-0.3682				

TABLE VI

t-Test for Muscle Diameter: TTX-Treated and Denervated Sides

Side	N	Mean	Standard Deviation	Standard Error	Variance	T Value	Degree of Freedom	Pr > /T/
Neur.	10	4.5663	±0.8994	0.2844	Unequal	-4.4181	17.0	0.0004
TTX	9	2.2923	±0.8034	0.2678	Equal	-4.3905	17.0	0.0004

TABLE VII

Summary of Histochemical Profile According to Myosin ATPase Activity

	Type I		Type II A		Type II B	
	ATPase Activity	Staining	ATPase Activity	Staining	ATPase Activity	Staining
pH 9.4	low	light	high	dark	high	dark
pH 4.6	high	dark	low	light	high	dark
pH 4.3	high	dark	low	light	low	light

Most muscles contain a randomly distributed mixture of fiber types, and their relative proportions may vary considerably between different muscles or even between different regions of the same muscle. For example, the mature soleus muscle, which was used as a reference in this study, had predominantly type I fibers with fewer types II A and II B (Fig. 8A,B). On the other hand, M. levator labii superioris, under normal physiologic conditions, differentiated into only type II A and II B and no type I fibers were observed (Fig. 9A,B). Biopsies from unoperated as well as saline-treated sides showed such differentiation into types II A and II B fibers (Fig. (A,B, 10A,B). After denervation and interference with impulse activity, the histochemical profile of the muscles markedly changed. Only muscle fibers of type II B were detected and the typical differentiation into two fiber types was absent (Fig. 11,12).

Figure 8 -- Serial sections of normal soleus from the rat.

A. Routine ATPase at pH 9.4, x545

B. Modified ATPase preincubated at pH 4.6, x545

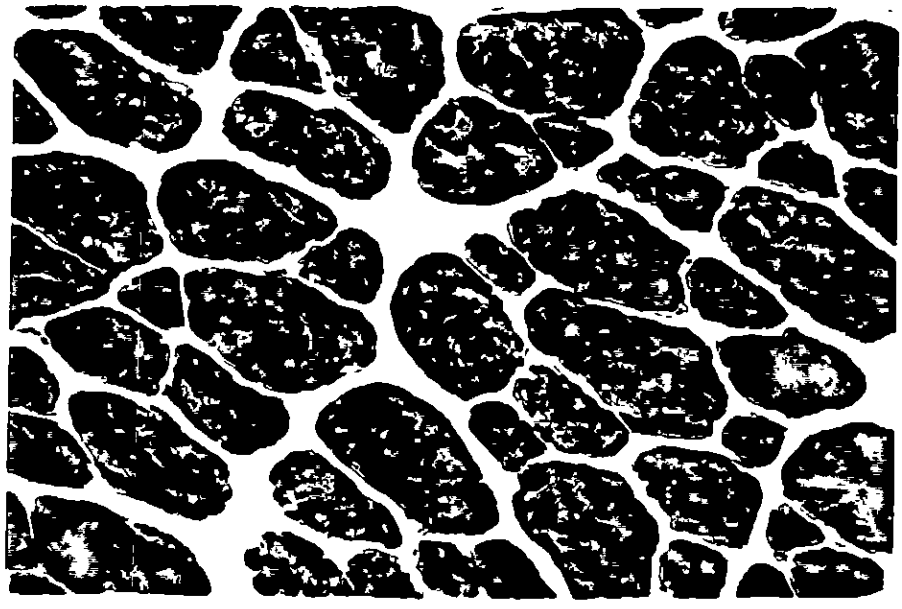




Figure 9 -- Serial sections of normal M. levator labii superioris from the rat (unoperated contralateral control side).

A. Routine ATPase at pH 9.4, x545

B. Modified ATPase preincubated at pH 4.6, x545



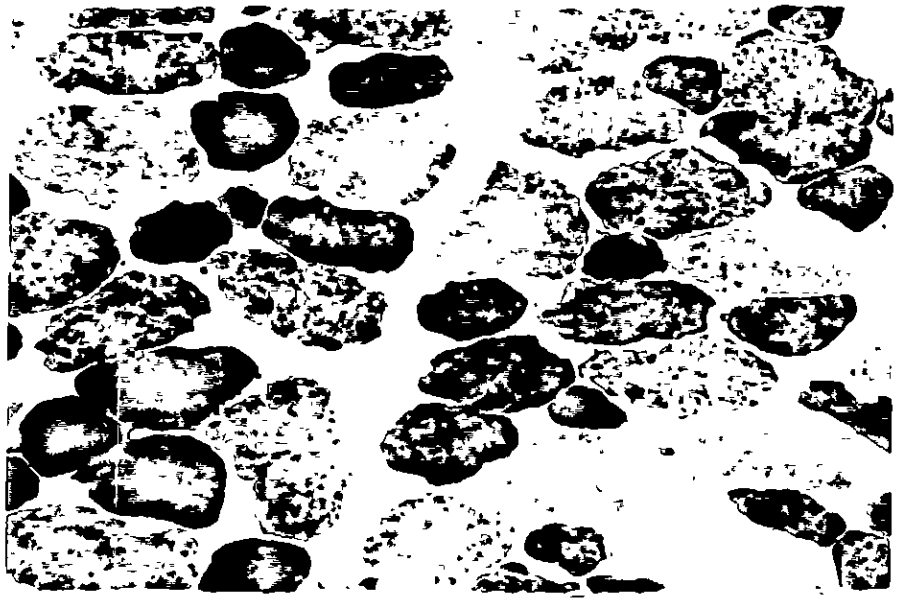
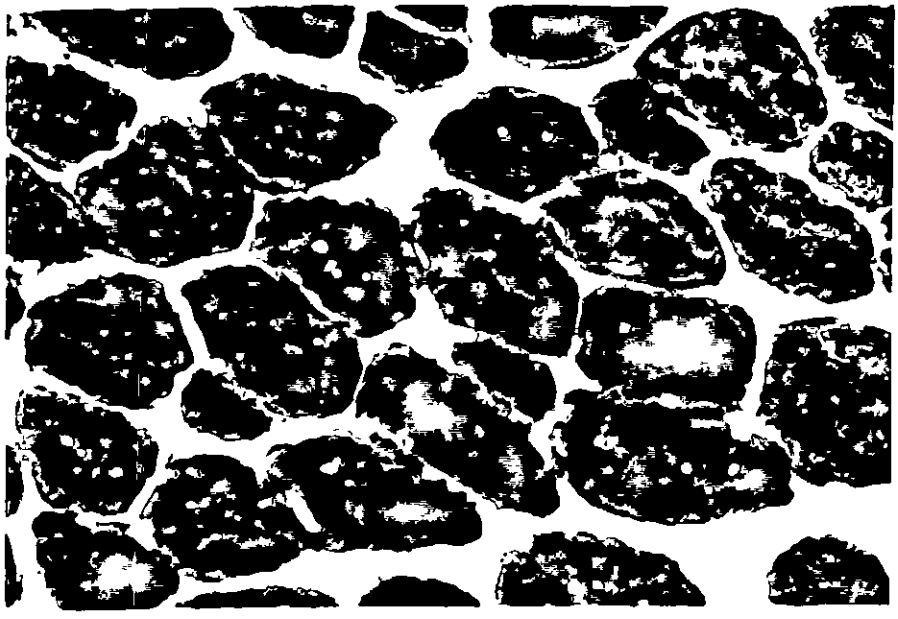


Figure 10 -- Saline-treated M. levator labii superioris

A. Routine ATPase, x545

B. Modified ATPase preincubated at pH 4.6, x545



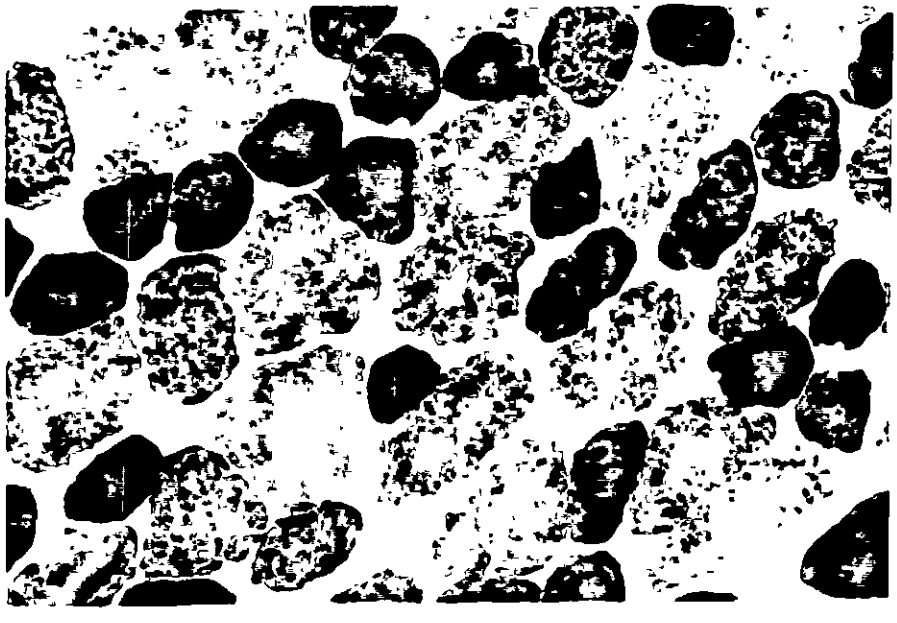


Figure 11 -- Denervated M. levator labii superioris.
Modified ATPase preincubated at pH 4.6, x545.

Figure 12 -- TTX-treated M. levator labii superioris
Modified ATPase preincubated at pH 4.6, x545

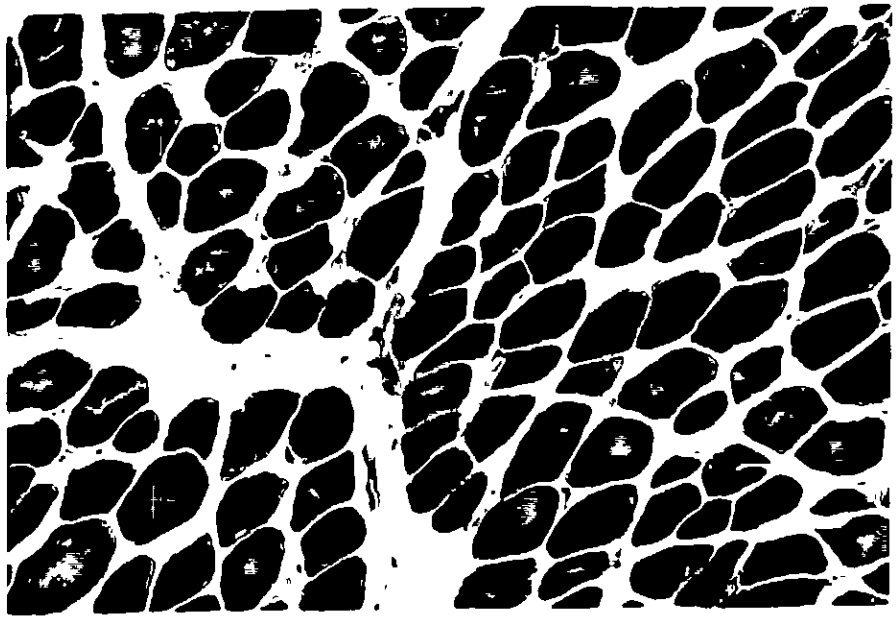




TABLE VIII

Summary of Fiber Types in the Three Sample Groups[1]

Group	Side	Type I	Type II A	Type II B
Group I (Saline)	Control	-	+	+
	Exp	-	+	+
Group II (Neurectomy)	Control	-	+	+
	Exp.	-	-	+(?)
Group III (TTX)	Control	-	+	+
	Exp.	-	-	+(?)

[1]Plus sign denoted presence and minus sign absence.

Morphometry of Bone

Appositional bone growth on the nasal process of the frontal bone was measured with an eyepiece micrometer in a fluorescence microscope. The numerical data were analyzed in the same manner as the muscle measurements. (1) Analysis of variance among the three unoperated sides showed no significant differences (Table IX). (2) Paired t-test was performed for intragroup comparison between unoperated and operated sides (Table X). The results showed no significant differences ($p < 0.05$) between unoperated and saline-treated sides. On the other hand, significant differences ($p < 0.05$) in both experimental groups were detected. Both TTX-treated and denervated sides showed decreased appositional bone growth compared with the unoperated, contralateral control,

TABLE IX

ANOVA for Skeletal Measurements: Unoperated Sides of the Three Groups

Source	Degree of Freedom	Sum of Squares	Mean Square	F Statistics	PR > F	R-Square	C.V.
Model	2	246.1901	123.0950	0.73	0.4969	0.0710	30.8751
Error	19	322.7027	169.6159				
Corrected Total	21	3468.8927					

TABLE X

Paired t-Test for Skeletal Measurements: Unoperated and Operated Sides Within Each of the Three Groups

Variables		Exp Side (unit)	Control Side (unit)	Difference (unit)	Mean difference	Standard error of Mean	T	Pr > /T/
Saline	1	33.5	34.5	-1.0	-4.3333	2.5314	-1.71	0.2291
	2	26.7	36.0	-9.3				
	3	32.0	34.7	-2.7				
TTX	1	30.0	30.7	-0.7	-7.3000	2.6420	-2.76	0.0246
	2	50.0	66.0	-16.0				
	3	35.3	52.3	-17.0				
	4	46.5	51.0	-4.5				
	5	43.3	57.3	-14.0				
	6	25.7	38.0	-12.3				
	7	59.0	53.0	6.0				
	8	23.5	29.0	-5.5				
	9	29.0	30.7	-1.7				
Neurectomy	1	39.3	58.7	19.4	-6.4100	2.6834	-2.39	0.0406
	2	18.7	24.3	-4.6				
	3	21.7	28.7	-7.0				
	4	49.0	59.7	-10.7				
	5	31.0	49.7	-18.7				
	6	30.7	34.3	-3.6				
	7	51.0	59.7	-8.7				
	8	41.3	34.0	7.3				
	9	32.0	29.7	2.3				
	10	35.0	36.0	-1.0				

[1] 1 unit = 8 μ

side. (3) Finally, the operated sides of the two experimental groups were compared by t-test (Table XI). Even though the mean value of TTX-treated sides were slightly greater than that of the denervated sides, there was no significant differences between the two at the 5% confidence level.

Nerve Histology

Serial cross-sections of those parts of the nerve fibers where the cuffs were applied were impregnated with silver protein and examined under the light microscope. Within the total sample, injury to the nerve was detected in only one control animal which showed partial axonal degeneration. Histology of the degenerated nerve fibers revealed some disruption of axons and connective tissue sheaths. Boundaries of fascicles were not clearly identifiable. Cross-sections of axons were of irregular size, probably as a result of swelling, construction and fragmentation of axoplasm as well as myelin. Also, schwann cells showed in the degenerating fibers had increased in number. Some of the Schwann cells appeared to have more than one nucleus in the hypertrophied cells (Fig. 14A, B). Histology of unaffected nerve fibers showed clear boundaries of fascicles and uniform size of cross-sectional areas of axons (Fig. 13A, B).

TABLE XI

t-Test for Skeletal Measurements: Denervated and TTX-Treated Sides

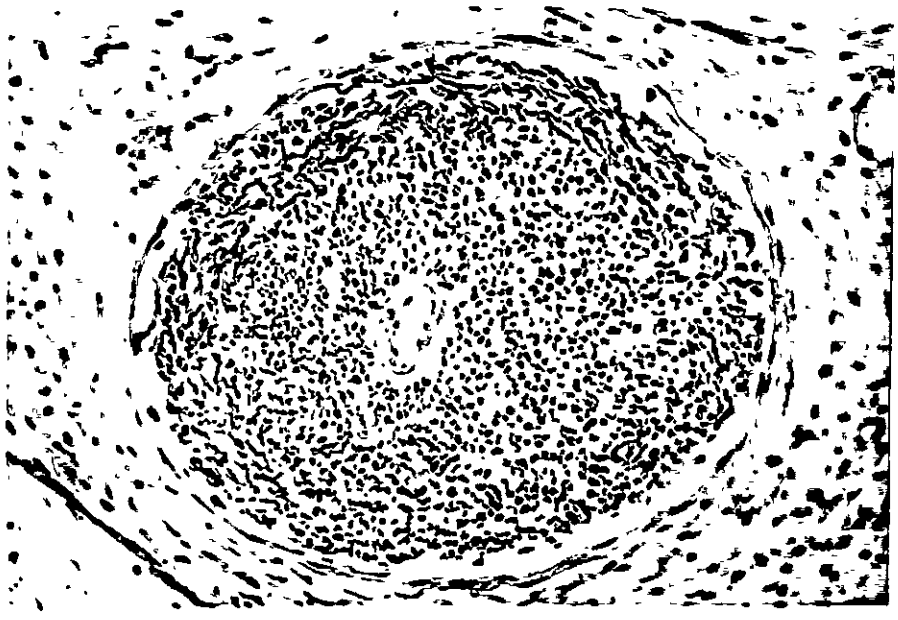
Group	N	Mean (unit)	Standard Deviation	Standard Error	Variance	T Value	Degree of Freedom	PR > F
Neurect.	10	35.0700	10.3527	3.2738	unequal	-0.5664	15.8	0.5791
TTX	9	38.0333	12.2431	4.0811	equal	-0.5717	17.0	0.5750

1 unit = 8 μ

Figure 13 -- Cross-section of normal facial nerve, silver protein staining (Bodian's method).

A. X218

B. X545



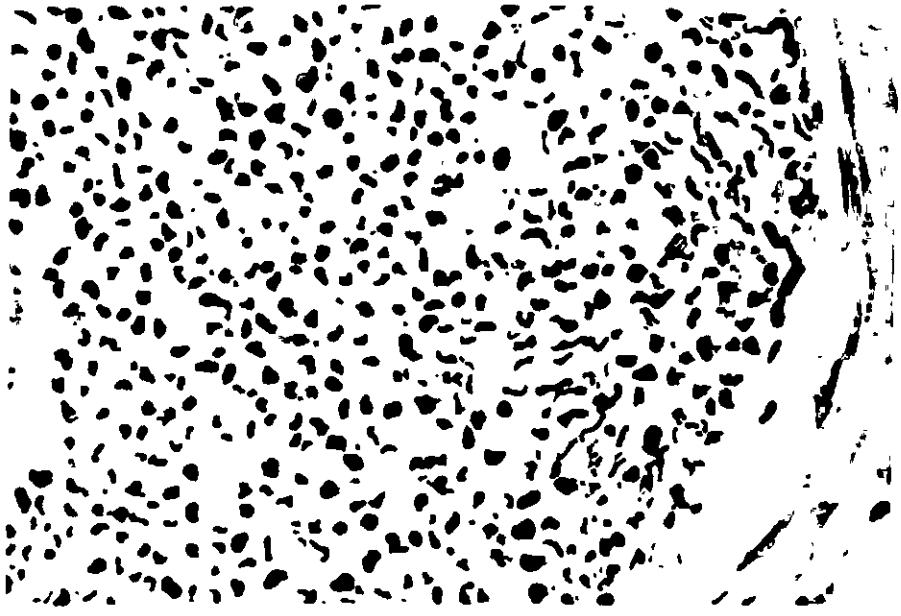
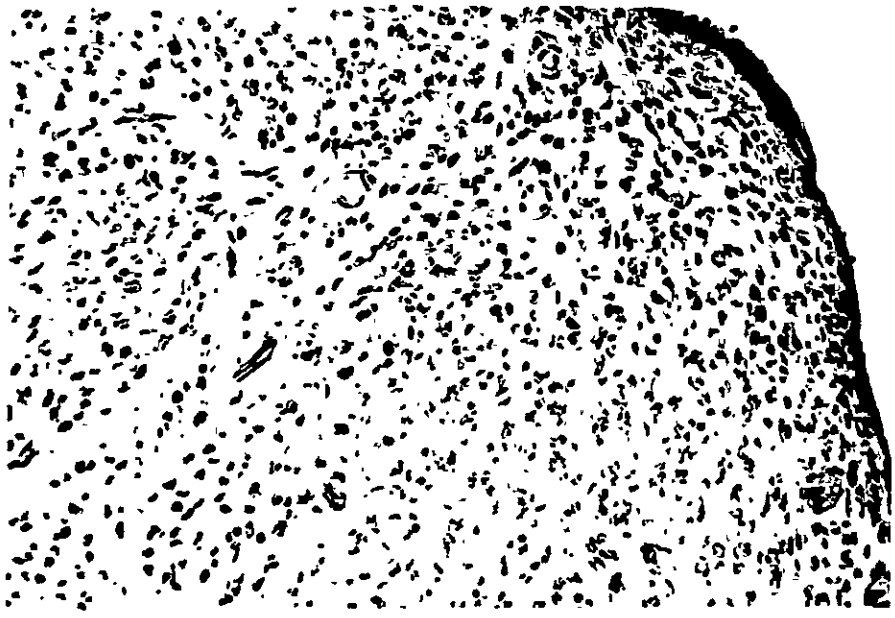


Figure 14 -- Cross-section of degenerating facial nerve silver protein staining (Bodian's method).

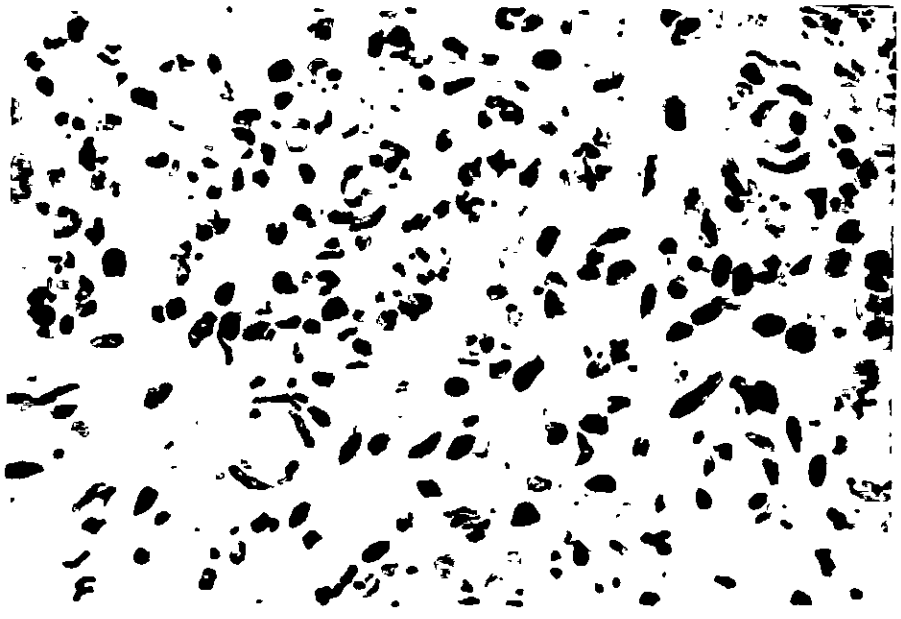
A. X218

B. X545

A



B



DISCUSSION

Although a number of investigators have shown neural influence on facial musculature and its secondary effect on skeletal tissue by the use of denervation experiments, strict dissociation experiments have not been used in order to separate and identify the respective roles of impulse activity versus axonal transport in craniofacial growth and development. Utilizing experimental methods designed to distinguish between these respective effects, the purpose of this study was to elucidate the differential influence of axonal transport and impulse activity on growth and maturation of facial musculature and its possible secondary function as a regulatory mechanism in the growth of underlying osseous tissue. A quantitative comparison between the effects of nerve impulse block with those of nerve transsection was made to determine whether factors different from impulse activity, i.e., axonal transport, play a role in the origin of denervation changes.

The techniques used in the past to dissociate the role of impulse activity and neurotrophic factors have been subjected to criticism. Incomplete loss of activity (e.g., tenotomy, cordotomy or immobilization experiments), arrest of axonal transport as well as conduction (e.g., local anesthetics) and partial denervation (pressure cuffs) have been common problems encountered in techniques attempting to achieve selective interference of impulse

conduction. Techniques in which axonal transport has been selectively abolished have also been used by many investigators (Albuquerque et al., 1972; Hoffmann and Thesleff, 1972; Cangiano, 1973). They have shown denervation-like changes in normally innervated muscles. Antimitotic drugs, such as colchicine or vinblastine, have been most commonly used in such experiments. However, interpretation of these results is necessaryily inconclusive because these drugs affect muscle cells by systemic direct action (Cangiano, 1973; Lømo, 1974).

In the present study, predetermined concentrations of tetrodotoxin (TTX) solution were utilized to achieve selective block of impulse conduction without affecting axonal transport. TTX specifically blocks the early inward sodium current by binding to some site in the sodium channel. The main reason for choosing TTX was that it has been convincingly demonstrated that it does not interfere with axonal transport (Ochs and Hollingsworth, 1971; Lavoie et al., 1977; Pestronk et al., 1976). As to the effectiveness of the TTX block, absence of vibrissa movement was used as an indicator. The animal sample in this study was divided into three groups; two experimental and one control. While the animals in one experimental group had neurectomy of the facial nerve, the animals in the other experimental group received TTX around the facial nerve resulting in blockage of impulse conduction. The control group was employed to control variables resulting from the surgical procedures, application of the device for drug

administration and systemic effect of the chemicals. The operations were performed only on the left side of the face for manipulative convenience and the right side was used as control side. Following surgery, the animals were separated from their foster mother then and then hand-reared for about two days. The animals seemed to develop normally and appeared healthy. There were no significant differences in weight gain among the three groups. The device for drug administration was designed for maximum but localized effect of toxin on the facial nerve. To minimize direct effect of toxin on the target muscle the cuffs were placed around the nerve (15-20 mm proximal to the target tissue), but occasional nerve damage from cuffs proved to be unavoidable. After careful examination, animals with evidence of nerve damage (non-movement of vibrissae or Wallerian degeneration seen in nerve histology) were excluded from the final results. Surgical wounds were healed completely in a couple of days, and no complications were encountered at any time during the experimental period.

Interpretation of data was based on (1) histochemical profile of M. levator labii superioris, (2) diameter of 200 fibers from randomly selected fields, and (3) amount of appositional growth on the anterior edge of frontal bone. The histochemical reaction for ATPase was used in the classification of fiber types. On the basis of intensity of ATPase activity at pH 9.4, muscle fibers are differentiated into types I and II, and further into types II A

and II B following preincubation at pH 4.6. Biopsies of M. gastrocnemius (GAST) and M. soleus (SOL), used as reference material, were processed simultaneously with M. levator labii superioris for histochemical reaction. GAST and SOL muscles demonstrated differentiation of fibers into types I and II, whereas normal M. levator labii superioris uniformly demonstrated only type II fibers.

Gauthier et al (1978) demonstrated the coexistence of slow (type I) and fast (type II) isoenzymes of myosin in all developing muscle cells which will mature into different types. Close (1969), Tyrer (1973) also demonstrated that mammalian muscle cells are all slow contracting at birth and become differentiated progressively thereafter and that a large increase in contraction speed occurs in those cells destined to be predominent in fast-type muscle cells. These observations suggest that different fiber types originate from a single cell lineage. Monster et al. (1978) examined 15 different human muscles and found that the relative proportion of type I cells was 20% made up the least-active muscles and reached a maximum of 85% in the most active muscles. This distribution suggests a strong functional/developmental role in determining particular differentiation patterns. The absence of type I fibers in M. levator labii superioris in the rat also appears to be due to developmental experience. Some unique function of muscle cells or impulse pattern generated from

their nerves might fail to develop type I fibers resulting, in permanent suppression of this fiber type.

A number of studies have demonstrated the considerable influence of the peripheral nerve in determining plasticity of fiber types in mature muscle. Buller et al. (1960) showed in the cat that crossing the nerves of a slow muscle such as the soleus and a fast muscle such as flexor hallucis longus, led to changes in contractile properties of these muscle, so that the slow soleus became fast under the new innervation, and the fast flexor hallucis slow. Application of histochemical techniques to this experimental model of cross-innervation in the cat and rabbit showed corresponding changes in fiber type patterns (Romanul and van der Meulen, 1967; Dubowitz, 1967). Thus the soleus, which normally contains predominantly type I fibers showed clusters of type II fibers after reinnervation by the nerve of the flexor hallucis longus, while the flexor hallucis or flexor digitorum, normally containing a predominance of type II fibers, showed large areas of type I fibers after innervation by the nerve of the soleus. Although Buller et al. (1960) attributed the changes in muscle properties following cross-innervation to dynamic characteristics dependent on chemical qualities of the respective nerve, they did not consider the possibility that the different nature of impulse-pattern in the two nerves might be a significant variable. A complete physiologic transformation of extensor digitorum longus (EDL) and soleus (SOL) of the rabbit after cross-innervation

occurs after only 15 weeks of chronic stimulation of EDL, which is naturally phasically activated, with tonic activity (10 Hz) such as is characteristically received by SOL (Lømo et al., 1974; Sréter et al., 1975). This result demonstrates the importance of pattern of muscle activity in determining the contractile properties of muscle. Rubinstein et al. (1979) examined the cells using fluorescein-labeled antibodies against the fast and slow myosins and suggested that the chronic stimulation of the muscle cells influenced a factor that alters gene expression resulting in transformation of fast myosin to slow myosin and vice versa.

In the present study, no differentiation into II A and II B histochemical fiber types occurred following denervation or interference with impulse conduction. Histochemically undifferentiated fibers showed uniformly high ATPase activity which indicated a strong reaction with ATP. Lopina (1976) has also reported increased ATPase activity by 1.5 times after denervation. The precise nature of the differentiation failure was not investigated but there seem to be two possible explanations. In young animals the first differentiation is into types I and II C fibers. As the animal matures, the type II C fibers differentiate successively into type II B and II A (Brooke, et al. 1971). Therefore, one possibility is that muscle fibers observed in the two experimental groups are developmentally as well as histochemically undifferentiated type II C fibers. On the other hand, a second possibility is that type II C fibers differentiated

only type II B fibers which demonstrate high ATPase activity after preincubation at pH 4.6. The question of histochemically undifferentiated fibers might have been clarified by additional preincubation of serial muscle sections at pH 4.3, in which type II B fibers show inhibited ATPase activity while type II C fibers show uninhibited activity. Regardless of the underlying basis for the failure of complete histochemical differentiation in the two experimental groups, which, in agreement with observations of other authors (Gutmann, 1976; Lømo et al., 1974; Romanul and Van der Meulen, 1967; Dubowitz, 1967), the present study shows that impulse activity plays a major role in the control of fiber type differentiation in the developing facial musculature of the rat into mature proportions of fiber types. However, the actual mechanism involved in transformation or differentiation of fiber types has still to be investigated. Of course, an electrical event itself is a factor that alters genetic expression to synthesize the specific proteins resulting in various types of muscle fibers. On the other hand, the possibility that chemical substances secreted from the nerve ending are directly involved in the transformation cannot be ruled out. Changes in the amount or characteristics of neurotrophic factors carried by axonal transport in response to neuronal impulse activity might reflect the production of isoenzymes or proteins. Also, inactive muscle cells, recognized as a pathologic stage by several investigators

(e.g., Deshpande, et al., 1976), might be completely or partly incapable of response to the trophic factors.

A second consideration in interpretation of the results concerns the evaluation of the separate roles of neurotrophic factors versus impulse activity on muscle fiber size. The diameters of muscle fibers were measured to quantify dimensional change. Diameters of muscle fibers in both the denervated and the TTX-treated muscles were significantly smaller ($p < 0.01$) than those of normal untreated muscles or saline-treated muscles. However, the diameters of muscle fibers in the TTX-treated muscle (21% smaller than normal) were significantly ($p < 0.01$) bigger than that of denervated muscles (46% smaller than normal). These data suggest that neuronal function, independent of impulse activity contributed to prevent part of the muscular growth failure after denervation in the growing rat. Provided no interactions are present it could be argued that axonal transport appears to be responsible for about 54% of total muscular growth failure following denervation, with the remaining 46% is due to disuse caused by impaired impulse conduction.

In an earlier study (Albuquerque et al., 1974), a silastic cuff mixed with 0.1% colchicine for selective inhibition axonal transport was used to examine neurotrophic influence on gastrocnemius muscle cells. A denervation-like phenomenon was observed in the muscle including muscle fiber atrophy. Although Albuquerque correctly attributed neurotrophic influence to the

basis for muscle atrophy, this cannot be conclusive from this experiment only. In addition to the direct effect of colchicine on muscle cells and its relative ineffectiveness in blocking axonal transport, interference of axonal transport for an extended period will affect integration of the axon and the amount of transmitter for normal impulse activity. Lømo and Rosenthal (1972) used a silastic cuff mixed with lidocaine to block impulse activity. They showed no significant difference in the amount of muscle atrophy between denervated and lidocaine-treated muscles and concluded that disuse caused by impaired impulse conduction is fully responsible for the denervated muscle atrophy. However, the interpretation of this finding is not convincing because of the indiscriminant nature of the lidocaine drug effect on the nerve.

Results similar to those of the present study have been reported by Davis (1981) for the limb muscle of rats. EDL muscles were denervated and/or immobilized for 7 days. Some of the rats received daily intramuscular injections of a proteinaceous extract of the rat sciatic nerve. Results showed that the decrease in size of immobilized muscles was significant but less than that of denervated muscles. Denervated muscles injected with nerve extract did not differ significantly from non-injected, immobilized muscles. The nerve extract used by Davis has been studied earlier in muscle cell culture and has been shown to promote the morphological maturation and maintenance of aneural

chick muscle cells (Markelonis and Oh, 1979) and to increase protein synthesis in postmitotic myotubes (Markelonis et al., 1980). Myotrophic material, an acidic glycoprotein purified from chick sciatic nerve, has been reported (Markelonis et al., 1979) with structural similarity to transferrin as evidenced by similarity in amino acid composition, amino acid sequence, immunological cross-reactivity, physiochemical parameters and iron-binding capacity (Markelonis et al., 1982). However, the increased blood level of transferrin after intravascular injection failed to show similar results as those following intramuscular injection of nerve extract (Davis, 1983).

With the foregoing studies in mind, the results of the present study suggest that trophic factors from nerve cells, although the chemical nature and exact control mechanisms have yet to be described, are necessary in conjunction with impulse activity for growth and maintenance of muscle cells. Neurotrophic substances must be responsible for the difference between the denervation and disuse (TTX-treated group) atrophies. However, the extent of the impulse activity on denervation atrophy cannot be defined from the present experiment. Changes in the amount of transported material in response to neuronal activity have been reported (Hendry et al., 1973; Schubert and Keutzberg, 1974; Schubert et al., 1976). Therefore, decreased axonal transport might be responsible for partial or even the total amount of disuse atrophy. For a better understanding of neurotrophic

control mechanisms on long-term maintenance or growth of muscle cells, future investigations should be focused on exploration of the chemical nature of myotrophic factors and their regulatory mechanisms at the molecular level.

Although investigations of neutotrophic influence on facial soft tissue growth have to date not been evaluated by direct measurements of the soft tissues, a couple of experiments have been performed to test neurotrophic regulation of craniofacial soft tissue matrices by measuring skeletal changes. These investigations (Behrents, 1975; Ghassemi-Tary et al., 1981) led to the conclusion that such an effect is small or negligible. However, if soft tissue measurements along with skeletal measurements were examined, the conclusion might have been different.

Skeletal measurements in the present study were taken to evaluate the secondary influence of an altered soft tissue environment on the underlying bone. Surface growth at the anterior edge of the frontal bone was assessed by measuring the distance from the periosteal surface of the external side to the DCAF line which represented ossification front level at experimental day 10. The results demonstrated that there was a significant ($p < 0.05$) decrease of surface appositional bone growth in both the denervated and TTX-treated groups compared to the controls. Further, the TTX-treated group had a greater mean value than the denervated group. However, statistical analysis failed to show significant differences between these two experimental

groups partly because of high values for group variance. The results suggest that loss of function and/or the smaller size of facial musculature were major factors in the decreased appositional growth of facial bones. The interpretation of the skeletal measurement was complicated by several factors which may explain the failure of showing differential secondary influence of neurotrophic functions on the osseous tissues. One of the factors would be the duration of the experimental period, which might have not been enough to separate results between the experimental groups. Longer experimental periods, therefore, might have disclosed less altered skeletal growth under the non-functioning muscles with intact neurotrophic factors compared to denervated muscles. Another factor would be the innervation by the trigeminal nerve of the skin and periosteum overlying facial bones. Normal development of the various soft tissues other than facial musculature as part of the facial draping might have masked relatively small differences in facial musculature between denervated and TTX-treated animals. The technical considerations regarding the morphometric technique used in this study might be still another factor. The frontonasal suture is irregularly angulated between the outer and inner surfaces of bones, which makes precise measurements of sutural width most difficult. A compromise was, therefore, made by measuring surface appositional growth. From each animal three right/ left side matched pairs of bone sections equidistant from the midline were used. An

increased number of sections might have decreased the value of the standard deviation within animals, which, in turn, could reduce group variance.

In conclusion, the present study was able to successfully dissociate trophic function of neural tissue from impulse activity and to show that such a function is a regulatory factor in the growth and maintenance of facial musculature. In the subject of craniofacial growth its existence has earlier generally been denied or only implicated in a speculative way. Methodologies employed in this study offer new approachs for further investigations on the relationship between neurotrophic functions and craniofacial growth. The composite data on soft tissue suggest that neurotrophic factors have a positive and independent role on growth and maintenance of muscle fibers, while impulse activity plays a major role, directly or indirectly in conjunction with neurotrophic factors, on differentiation of fiber types. Also, the data on skeletal measurements suggest secondary influences on osseous tissue, but the interpretation of these results is not totally conclusive. However, they tend to support the concept of facial osseous growth dependency on the soft tissue draping. Experiments on skeletal tissue need to be designed with longer experimental periods and with already identified alterations in the morphometric technique. Further studies in neurotrophic control mechanisms now need to be advanced into the molecular level. Identification of the chemical nature of neurotrophic

substances will be important in attempts to understand the nature of their control actions and molecular modification for gene expression in the normal growth process and also developmental sysplasias in the craniofacial complex. These considerations are now necessary at the hypothetical level.

SUMMARY AND CONCLUSIONS

Many previous studies have attempted to demonstrate that neurotrophism represents an important process in the control mechanism in the craniofacial growth. For many reasons, however, past investigations have been unable to distinguish the respective effects of neurotrophic factors carried by axonal transport and those related to nerve impulse transmission. The objective of this study was (1) to develop an experimental method that makes possible determinations of the separate growth control effects of neurotrophic factors versus nerve impulse transmission, and (2) to employ this procedure in determining their respective growth regulatory roles in growth and maturation of the facial musculature and skeletal tissue.

Using very young growing rats one experimental group received unilateral transsection of the facial nerve in order to determine the effects of simultaneous absence of both axonal transport (neurotrophic factor) and nerve impulse transmission. In a second experimental group only nerve impulse activity was blocked by means of tetrodotoxin diffusion. The results, therefore, demonstrated only the neurotrophic influence in the growth process. Data interpretation was based on diameters of muscle fiber, histochemical profiles of muscle biopsies and extents of bone growth in the experimental animals compared to controls.

Results of this study may be summarized as follows:

(1) Fiber diameters of denervated and paralyzed (TTX-treated) muscles were significantly different from those of normal muscle. The greater difference was observed in the denervated muscles which fibers were 46% smaller ($p < 0.01$) in diameter than in normal muscle. Paralyzed muscle fibers (TTX-treated) showed less severe growth failure but were still 21% smaller ($p < 0.01$) than those of normal muscle. Also, fibers of the denervated muscles were significantly smaller ($p < 0.01$) than TTX-treated fibers.

(2) Histochemistry of M. levator labii superioris from both saline-treated and unoperated sides demonstrated differentiation into type IIA and IIB fibers. However, fibers from TTX-treated and denervated muscles remained undifferentiated and did not show segregation into mature histochemical patterns.

(3) Skeletal measurements revealed reduced appositional growth of underlying bone in both denervated and paralyzed animals compared to controls ($P < 0.05$). Even though the mean amount of bone apposition was somewhat greater in the paralyzed animals, there were no statistically significant differences in bone apposition between the two groups.

In conclusion, the results of this study indicate 1) that axonal transport as well as impulse activity play important roles in growth and maintenance of facial musculature in the growing

rat, 2) that impulse activity is a decisive factor in determining changes in the histochemical profile of muscles, and 3) that impaired function and/or size of facial muscles secondarily affect appositional growth of the facial bones. Denervated and paralyzed muscle did not show significant differences in their influence on bone growth but a longer experimental period may possibly demonstrate that such differential influences do, in fact, exist.

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