DEGENERATION OF A MUSCLE FOLLOWING REMOTE NERVE DAMAGE: PHYSIOLOGICAL CHANGES AND TRIGGERING MECHANISMS

by

Kirkwood Ely Personius, Jr.

A Dissertation Submitted to the Faculty of the

INTERDISCIPLINARY PROGRAM IN PHYSIOLOGICAL SCIENCES

In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

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and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy

R F. Chap Reginald F. Chapman, Ph. D.	7/16/98 Date
Douglas D Stuart G Douglas G. Stuart, Ph. D.	7/16/98 Date
Andrea Yool Andrea J. Yool, Ph. D.	7/16/98 Date
Richard B. Levine, Ph. D.	7/15/98 Date
	Date

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

R. F Chap
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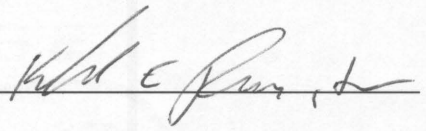
Signed: 

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I would like to dedicate this work to the late, Edmund Arbas, who died at too young of an age. I hope I have carried-out this project in a way fit to your memory.

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ABSTRACT

Muscle depends on innervation and contraction to maintain a differentiated state. Denervation or disuse, therefore, often leads to muscle atrophy. In grasshoppers, muscle degeneration can also be induced when a hindlimb is lost by autotomy. In this case, the thoracic muscles which degenerate are neither damaged nor denervated, suggesting the existence of transneuronal mechanisms that influence muscle survival. Arbas and Weidner (1991) found that muscle degeneration is induced when the leg nerve (which does not innervate the thoracic muscles) is severed during autotomy.

To characterize this autotomy-induced degenerative process, I studied a thoracic tergotrochanteral depressor muscle (M#133b,c) subsequent to autotomy in the grasshoppers, *Barytettix psolus* and *B. humphreysii*. The degeneration of M#133b,c is generally complete by 15 days after autotomy, when muscle cross-sectional area is reduced to 4% of the contralateral-control. The rate of muscle degeneration is initially slow, but at ~10 days post-autotomy, degeneration becomes rapid and muscle fiber number becomes reduced. During this rapid phase, degeneration of M#133b,c occurs by programmed cell death. Indicators of programmed cell death-up-regulation of ubiquitin-immunoreactivity, condensed nuclear chromatin and DNA fragmentation-are all present 10 and 15 days post-autotomy. The rapid phase of M#133b,c degeneration is also characterized by 'denervation-like' changes in fiber electrophysiology including depolarized resting membrane potentials, post-inhibitory rebound, smaller evoked

excitatory junctional potentials and an increased frequency of spontaneous miniature potentials.

The initial trigger for autotomy-induced muscle degeneration appears to be the loss of some proprioceptive input, since severing of afferents from the subgenual organ leads to muscle degeneration. The loss of exteroceptive chemo or mechanoreceptor input, however, does not lead to degeneration. Autotomy is also correlated with a decline in activity in the nerve innervating M#133b,c during the period of programmed cell death. The population of active motor units within the nerve is also decreased during this period.

These findings suggest the transneuronal mechanisms that influence muscle degeneration following autotomy. The loss of proprioceptive input probably leads to deafferentation of motoneurons within the metathoracic ganglion. The deafferentated motoneurons have a decreased level of spontaneous activity which triggers the subsequent muscle degeneration by programmed cell death.

CHAPTER ONE: LITERATURE REVIEW AND INTRODUCTION

Literature review

When a human suffers a cerebral vascular accident, the primary motor cortex often does not receive adequate blood supply, and consequently the axons descending from the motor cortex to the motoneurons of the brainstem and spinal cord will die. Patients with stroke often experience weakness and atrophy of muscles on the side opposite the injury, which was thought to be secondary to disuse, alteration in blood supply, or arthropathy (Namba, Schuman & Grob, 1971). Upon closer inspection, however, many of the affected muscle fibers demonstrate changes similar to those seen after denervation; (i. *e.* fibrillations; Goldkamp, 1967). After a stroke, why would muscles fibers behave as if denervated when a cerebral vascular accident effects neurons within the motor cortex, but not the motoneurons which directly innervate muscle fibers? An answer was proposed by McComas *et al.* (1973) when they demonstrated that the number of motor units on the side of weakness was reduced to half the normal number by two months post-stroke. They concluded that degenerative changes in motoneurons of the spinal cord, possibly secondary to the loss of trophic input from the motor cortex, led to muscle atrophy after stroke. Thus, damage in one area of the nervous system can have far reaching effects along synaptically connected pathways.

Denervation-induced muscle atrophy also occurs in amyotrophic lateral sclerosis. The initial site of injury is unknown, but the effects extend throughout the CNS. For

example, Betz cells are lost in the motor cortex, the corticospinal tract degenerates, and motoneurons degenerate within the spinal cord leading to muscle atrophy (Appel, 1981). Similar transsynaptic effects are proposed in Alzheimer's disease, since the anatomical areas of degeneration occur along synaptically connected pathways (Pearson and Powell, 1989). From these examples of human disease, it is clear that understanding the cellular mechanisms of regulatory communication between nerves and their targets is fundamental to understanding how the neuromuscular system develops and is maintained, and how breakdown of these interactions leads to disease. My dissertation addresses this area of neurophysiology.

The dependence of neurons upon afferent input and feedback from their targets has been well documented during both development and maturity (Hubel *et al.*, 1977; Oppeinheim and Havekamp, 1988; Deitch and Rubel, 1989a, b; McMannaman *et al.*, 1990). During vertebrate development, large numbers of motoneurons die due to inadequate trophic support from their targets (reviewed in Oppenhiem, 1991), and during this developmental period, motoneurons may be rescued by muscle extracts (McMannaman *et al.*, 1990). The loss of various identified trophic factors, isolated from many neuronal and non-neuronal tissues, also affects neuronal survival. Motoneuron survival during development is supported by many trophic factors (reviewed by Henderson, 1996). These include members of the neurotrophic family [nerve growth factor (NGF) (Li *et al.*, 1994), brain-derived neurotrophic factor (BDTF) (Freidman *et al.*, 1995), neurotrophin-3 and neurotrophin-4/5 (NT-3 and NT-4/5) (Glass and Yancopoulus, 1993], the neurokines [ciliary neurotrophic factor (CNTF) (Dechiara *et al.*, 1995),

leukemia inhibitory factor (LIF)] the transforming growth factor family (TGF-ß), glial cell line derived neurotrophic factor (GDNF) (Sagot, 1996; Henderson *et al.*, 1994), insulin-like growth factor 1 (IGF-1) (Recio-Pinto *et al.*, 1986), and Cardiotrophin-1 (Pennica *et al.*, 1996). Furthermore, many of these trophic factors are present at appropriate stages in the limb bud and spinal cord development when target-derived factors are thought to be necessary for motoneuron survival. These include BDNF, NT-3, NT-4, GDNF and Cardiotrophin-1 (Vejsada *et al.*, 1995). The development of a neuron is also dependent upon correct input. For example, change in sensory input to the visual neocortex leads to significant changes in the formation of ocular dominance columns (Hubel *et. al.*, 1977; Fox and Daw, 1993).

During adulthood, neurons remain dependent on their targets and inputs. Mature neurons have been shown to atrophy following the death of their targets (Bensholom and White, 1988; Oppenheim and Haverkamp, 1988), or following deafferentation (Deitch and Rubel, 1989a,b). Many of the trophic factors found to rescue motoneurons from death during development also prevent atrophy of motoneurons following axotomy (Li *et al.*, 1994). Mature neuronal properties can also be regulated by the activity of their target (Foehring *et. al.*, 1978a,b; Oppenheim and Haverkamp, 1988), while removal of motoneuron input leads to changes at the neuromuscular junction, such as depressed synaptic transmission to muscle, and decreased post-synaptic potential amplitude and speed (reviewed by Titmus and Faber, 1990).

Similar interactions occur between neurons and muscles. The development of primary muscle fibers is dependent upon innervation, and the muscle requires

suprathreshold activity for survival (Ashby *et al.*, 1993). Activity may regulate primary muscle fiber survival by modulating the levels of neuregulin at the neuromuscular junction (Trachenberg, 1998), while secondary muscle fiber number may ultimately depend upon primary muscle fiber survival (Ashby *et al.*, 1993). Neuronal activity is also important during the period of muscle fiber synaptic elimination (Balice-Gordon and Lichtman, 1994), and the overproduction of muscle-derived GDNF has been shown to prolong the duration of muscle fiber hyperinnervation (Nguyen *et al.*, 1998). Calcitonin gene-related peptide (CGRP), which is co-released with acetylcholine from motoneurons has multiple effects on muscle, including induction of new nicotinic acetylcholine receptors in cultured muscle cells (Fontaine *et al.*, 1987), increasing muscle tension (Takami *et al.*, 1985) and twitch potentiation (Takamori and Yoshikawa, 1989). CGRP is present in motoneurons post-natally, but levels decline with neuromuscular junction maturation (Popper *et al*, 1992). In adults, cross innervation studies have shown that innervation influences motor unit properties (Foehring *et al.*, 1987a,b), muscle biochemical properties (Buller *et al*, 1969), and ultimately muscle contractile properties (Buller *et al.*, 1960). Mature muscles also affect the motoneurons which innervate them, since NT-4, a target-derived factor from muscle, has recently been shown to affect adult motoneuron plasticity (Funakoshi *et al.*, 1995).

Invertebrate neuronal development and maintenance are controlled by cellular interactions similar to those described above. During development, competitive interactions regulate neuron number (Donaldson and Josephson, 1981; Murphey, 1986), and a neuron's properties are regulated by synaptic inputs (Loer and Kristan, 1989). For

example, deafferentation of motoneurons reduces dendritic growth in developing insect nervous systems (Murphey *et. al.*, 1976), while the accumulation and proliferation of myoblasts during metamorphosis is dependent upon innervation (Consoulas and Levine, 1997). Additionally, survival of the muscles used during eclosion of adult insects is dependent upon hormone titer and neuronal activation (Lockshin, 1981; Schwartz, 1993). In adult animals, muscle cell phenotype is transformable and dependent upon neuronal impulse activity (Mellon and Quigley, 1988), and activity of motoneurons has been shown to regulate transmitter release, fatigability and morphology of neuromuscular synapses (Lnenicka and Atwood, 1985). Unlike the neurons of vertebrates, some invertebrate neurons appear able to survive independently of their targets and persist after removal of their targets by surgery (Whitington *et al.*, 1982), or normal degeneration of the target during adulthood (Arbas and Tolbert, 1986).

The identification of trophic factors is just beginning in invertebrates. Cultured embryonic *Drosophila* cells have been shown to produce a factor that stimulates neurite outgrowth from embryonic chick ganglia. Outgrowth of neurites is blocked by antibodies to mouse NGF, suggesting an immunological relationship between the *Drosophila* factor and mouse NGF (Hayashi *et. al.*, 1992). Additionally, a receptor tyrosine kinase which is similar in sequence to the neurotrophin receptor tyrosine kinases has be found in *Drosophila* (Wilson *et al.*, 1993) and these receptors are found in the developing nervous system (Oishi *et al.*, 1997). Insulin-like receptors have been also found at the neuromuscular junction of developing *Drosophila* (Gorczyca *et al.*, 1993).

Arbas and colleagues have developed an insect model of neuromuscular synaptic plasticity and muscle degeneration following damage to a remote nerve. The relative simplicity of insect anatomy offers the advantage of studying *in vivo* changes of identified neurons and synaptic connections. In addition, the cellular building blocks that construct the neuromuscular system are conserved through the evolutionary process (Arbas *et al.*, 1991). Therefore, the cellular processes demonstrated in this dissertation may be common across animal species, and the knowledge produced will be pertinent to understanding the cellular communication between nerves and their targets in invertebrate and vertebrate systems.

Introduction to grasshopper model

Grasshoppers, like a number of vertebrates and invertebrates, are able to spontaneously drop or autotomize an appendage for defensive purposes (McVean, 1975). The plane of autotomy is between the trochanter and the femur, and the leg does not regenerate after loss. Autotomy severs the nerve running into the hindlimb (N5), but no muscle damage occurs, since no muscles span the plane of autotomy. Arbas and Weidner (1991) found that in the grasshopper, *Barytettix psolus*, innervated and completely undamaged thoracic muscles that are normally involved in moving the leg exhibited severe degeneration to <15 percent of their normal mass by 42 days after autotomy. Furthermore, muscle degeneration was restricted to the side of autotomy. Experimental cutting of the N5 and loading/unloading of the muscles showed that degeneration of these

undamaged thoracic muscles was triggered transneuronally as a result of severing N5. The affected thoracic muscles operate the proximal two leg segments (coxa and trochanter), which remain in place after autotomy and are innervated by nerve 3 or nerve 4 (N3, N4). Six muscles compose this group: the tergal and pleural promoters of the coxa (M#188 and 128), tergal and pleural remoters of the coxa (M#119, 120 and 129) and the depressor of the trochanter (M#133b,c) (all muscle and nerve nomenclature is after Snodgrass, 1928). All of these muscles do not degenerate to the same extent (Clinton and Arbas, 1994), and the variation may be related to innervation and muscle fiber type (Clinton and Arbas, 1995). Staining the motoneurons innervating M#133b,c and M#120, by backfill techniques, has shown that they maintain terminals to their targets long after muscle atrophy is complete (Villalobos and Arbas, 1988; Clinton and Arbas, 1994). The persisting motoneurons, however, demonstrate significantly atrophied somata and reduced dendritic arbors.

Effects of autotomy upon neurons with axons in nerve 5

What neuronal axons are severed when the hindlimb is autotomized, and how might the circuitry of the metathoracic ganglion be affected? N5 carries both afferent and efferent information. Afferent information is derived from two sources: the exteroceptors and the proprioceptors of the hindlimb. The exteroceptors include the many mechano- and chemosensory sensilla that are distributed along the surface of the limb, but especially on the tarsus (Laurent and Burrows, 1988). The mechanoreceptors respond to

movement with phasic bursts of activity and their axon terminals form a mapped representation of the limb in the ventral neuropil (Burrows, 1992). The proprioceptors include the chordotonal organ and campaniform sensilla that monitor position and movement of the tibia and tarsus (Laurent, 1987), the subgenual organ, Brunner's organ and the lump receptor of the femur as well as the many proprioceptors of the coxo-trochanteral joint (Bräunig and Hustert, 1985a). All of these proprioceptors are involved in coordinating posture, walking, jumping and defensive kicking (Heitler and Burrows, 1977a, b, c; Bräunig and Hustert, 1985b).

Efferent information is carried by three groups of neurons; motoneurons, common inhibitory motoneurons (CI) and dorsal unpaired medial (DUM) neurons. Six muscles are intrinsic to the autotomized lower leg and they are probably innervated by 20-23 neurons, thirteen of which have been identified (Burrows and Hoyle, 1972; Heitler and Burrows, 1977b; Phillips, 1980; Laurent and Hustert, 1988). The CI motoneurons are multiaxonal and primarily co-innervate muscle fibers along with slow excitatory motoneurons (Burrows, 1972, Hale and Burrows, 1985). These CI motoneurons are thought to be involved in allowing quick transition from static posture to rapid movement (Wolf, 1990). The neuromodulatory DUM neurons have bilateral axons and can be either mono- or multiaxonal. Three identified DUM neurons have axons within N3, N4 and N5, and therefore, may be important in autotomy-induced muscle degeneration (Campbell *et al.*, 1995).

Connections within the metathorcic ganglion

How are the several thousands of afferent and tens of efferent neurons running in N5 connected in the metathoracic ganglion? Mechanosensory exteroceptors primarily connect with motoneurons via spiking and non-spiking local interneurons (Burrows and Siegler, 1982; Burrows and Siegler, 1976; 1979). Direct connections from exteroceptors to motoneurons are rare. Afferents to spiking local interneurons are excitatory, and spiking local interneurons may have either excitatory or inhibitory connections to non-spiking interneurons, intersegmental interneurons or motoneurons. Afferents to non-spiking interneurons are also excitatory and non-spiking interneurons may have inhibitory connections to other non-spiking interneurons, or either excitatory or inhibitory actions on intersegmental interneurons or motoneurons (Burrows and Siegler, 1978; Laurent and Burrows, 1988; Burrows, 1992). Proprioceptors have the same types of connections as exteroceptors, except that direct connections to motoneurons are more common (Laurent and Hustert, 1988; Burrows, 1992). The web of inhibitory interactions between interneurons and their targets ensures that individual interneuronal response is in context with the activity of the entire body. All known connections, except those with intersegmental interneurons, are ipsilateral. Afferent connections to interneurons and motoneurons are parallel and distributed, with afferents synapsing upon many interneurons and interneurons synapsing upon many motoneurons (Laurent, 1986; Burrows, 1992).

Possible triggering mechanisms of autotomy-induced muscle degeneration

What cellular mechanisms may be responsible for initiating muscle degeneration after local damage of N5, since in at least two of the affected muscles innervation appears to be maintained? There appear to be six possibilities:

1) Thoracic muscles may degenerate due to disuse after loss of the hindlimb.
2) Degeneration may occur secondarily as a result of trans-neuronal changes in the neurons to the affected muscle, leading to denervation atrophy.
3) Multiaxonal neurons with branches in N5 as well as N3 or N4 may be broken by autotomy and could carry a damage signal directly to the muscle.
4) The breakage of N5 would axotomize motoneurons that activate the distal leg muscles. Axotomized motoneurons undergo many changes that may have retrograde influences on the central neurons within the metathoracic ganglion, eventually affecting the thoracic motoneurons and their muscles.
5) Severing the axons of sensory neurons in nerve 5 may induce changes through deafferentation of central neurons, including the motoneurons of the affected thoracic muscles.
6) During development in some insect species, hormones control muscle and nerve degeneration. If autotomy leads to changes in hormonal titer, a humoral factor could act as an intermediary to degeneration following damage to N5.

Each of these six possible mechanisms will be discussed in turn.

Muscle atrophy following disuse

Muscle disuse is characterized by a variable rate of atrophy and transient small changes in fiber electrophysiology (reviewed by Musacchia *et. al.*, 1988). Following disuse the ratio of muscle strength to cross-sectional area remains normal and no decrease in fiber number is seen after cast immobilization (Cardenas *et al.*, 1977). Animal models of disuse include, immobilization via internal joint fixation or casting, tenotomy, weightlessness, nerve blockage, and isolated spinal cord segment (reviewed by Gutmann, 1976). The resulting rate of disuse atrophy appears to vary considerably, perhaps because so many different animal species and different models have been used to study disuse atrophy (Fishbach and Robbins, 1971; Michel and Gardiner, 1990). However, variation in rate of atrophy is still seen even when the same species, muscle and model of disuse are studied (Flynn and Max, 1985; Michel *et al.*, 1990). The resting length of muscle fibers is one of the determinants of the time course of disuse atrophy. Muscles immobilized in a stretched position actually add sarcomeres and are slow to atrophy, while those fixed in a shortened position lose sarcomeres and atrophy more quickly (Tarary *et al.*, 1972). In general after disuse, slow-twitch fibers atrophy more quickly than fast-twitch fibers (Jaffe *et al.*, 1978), while the opposite is true after denervation (Herbison *et al.*, 1979). This appears logical, since slow-twitch fibers receive continual neuronal input in order to maintain the animal in a weight-bearing posture, while fast-twitch fibers are recruited only during more intense movements (Saltin and Gollnick, 1983). The mechanism for loss of strength after disuse is unknown, but is thought to be due to a decrease in protein

synthesis and an increase in protein degradation in the muscle (Goldspink *et al.*, 1983). If disused muscles are subsequently denervated, the rate of protein breakdown rapidly increases with little or no change in synthetic rate (Goldspink *et al.*, 1983)

Brooks (1970) studied physiological changes accompanying disuse atrophy in a predominately fast-twitch muscle, the cat gastrocnemius. No significant changes where seen in fiber resting membrane potential after one month of disuse, though a small reversible fall in resting membrane potential was seen in the first week. No changes were seen in the spatiotemporal response of the action potential, rheobase or cell input resistance. Fischbach and Robbins (1971) also found no significant change in resting membrane potential, cell input resistance or miniature end-plate potential frequency in immobilized rat soleus muscle, composed primarily of slow-twitch fibers. They did, however, find a transient increase in extrajunctional sensitivity to transmitter.

The effects of disuse in muscles of invertebrates are similar to vertebrate animals. Anderson (1979) studied the effects of disuse of the retractor unguis, in cockroach after removing the pretarsus of the leg. He described breakdown of myofibrils, sarcoplasmic reticulum and mitochondria, but these findings were only apparent after 9 weeks of disuse. Jahromi and Bloom (1979) describe ultrastructural changes in a leg muscle of the desert locust (*Schistocera gregaria)* following tenotomy or joint immobilization. After tenotomy, a few fibers showed structural changes, including streaming Z-discs and disrupted myofibrillar structure and vesiculated mitochondria as early as 9 days after the operation. By 45-73 days following tenotomy, some atrophying fibers contain large membranous complexes containing loosely packed whorls of membrane, and at this time

myofilaments began to disappear in some fibers with lipid replacement. Interestingly, the whorls of membrane found by Jahromi and Bloom (1979) are similar to those found by Rees and Usherwood (1972) 68 days following denervation of locust muscle, suggesting a similar degeneration process occurs at long periods following injury. Less severe changes were found after immobilization than after tenotomy, as occurs in vertebrates. Similar ultrastructural changes following disuse are seen in crayfish muscle fibers (Jahromi and Atwood, 1977), where disuse atrophy is not accompanied by changes in electrical properties (Atwood *et al.*, 1978).

Arbas and Weidner (1991) used three methods to determine whether muscle disuse was the cause of muscle degeneration after autotomy in the grasshopper. 1) They autotomized a hindlimb, severing N5 and re-loaded the proximal joints by re-attaching the limb with cyanoacrylate. Degeneration occurred as after autotomy alone. 2) They cut the tendon of the tibial extensor muscle through a small hole in the cuticle. The leg became inoperable for walking or jumping, and the animal held the limb next to its thorax, off the ground. No degeneration occurred 30 days following tenotomy, even though the muscles that depress the coxa and trochanter were unloaded when the animal held the limb off the ground. 3) They surgically severed N5 through a window in the thoracic cuticle. Again, the hindlimb was inoperable and held in a similar position, next to the thorax, unloading the coxal and trochanteral depressors. Degeneration occurred as after autotomy. Arbas and Weidner concluded that damage to N5 transneuronally induces atrophy of the thoracic muscles, and that muscle degeneration post-autotomy was not a disuse response.

Muscle atrophy following denervation

Muscle atrophy following denervation is characterized by a moderate rate of atrophy and by significant changes in muscle electrophysiology. Early after denervation, no muscle fiber loss is observed (1 mo), but at longer times fiber loss occurs and fibers are replaced by fat, a process called fatty necrosis (reviewed by Grinnel, 1994). Decreases in fiber diameter are found in most vertebrate muscles soon after denervation (reviewed by Gutmann, 1976). Transient hypertrophy of muscle is seen after denervation of avian and mammalian diaphragms due to stretching of the muscle by the rib cage (Stewart, Sola and Martin, 1972). Rees and Usherwood (1972) also found that transient hypertrophy occurs in the locust (*Schistocerca gregaria*) retractor unguis muscle 4 days after denervation, but by 20 days muscle cross-sectional area had decreased to 35 percent of normal. Reduction of fiber size resulted from a loss of protein contractile filaments and a degeneration of muscle organelles (Rees and Usherwood, 1972).

The loss of muscle protein after denervation occurs primarily through increased rates of protein breakdown associated with a non-lysosomal ATP-dependent proteolytic process in rat (Medina *et al.*, 1995); at the same time, the rate of protein synthesis is slowed. Levels of Id-1 mRNA, a negative regulator of transcription, has been found to increase 2- to 7-fold in rat 10 days following denervation, suggesting a mechanism for the cell's diminished ability to produce new proteins (Gundersen and Merlie, 1994). Trachtenberg (1998) has demonstrated that muscle fiber nuclei undergo PCD after neonatal denervation, while Wing, Haas and Goldberg (1995) have demonstrated an up-

regulation of ubiquitin in muscle following two days denervation in rat skeletal muscle, suggesting that muscle degradation occurs as a consequence of programmed cell death by the 26S proteasome complex.

After denervation, vertebrate muscle fibers show a range of physiological changes: a reduction in RMP, increased specific membrane resistance (Redfern and Thesleff, 1971), increased sensitivity to extrajunctionally applied transmitter (Axelsson and Thesleff, 1959), muscle fibrillation starting from spontaneous membrane potential changes (Thesleff and Ward, 1975), appearance of tetrodotoxin resistant action potentials (Thesleff, 1974), and presence of post-inhibitory rebound (Marshall and Ward, 1974). Similar changes also occur in invertebrates. Locust (*Schistocerca gregaria*) extensor tibiae muscle fibers depolarize between 10 and 18 days post-denervation (Usherwood, 1963a). Extrajunctional sensitivity to L-glutamate was seen to increase in locust muscle 10-14 days after denervation (Gration, Clark and Usherwood, 1978). Impulse transmission failed between 9 and 24 days following denervation of locust muscle. Shortly after synaptic transmission failure, spontaneous membrane potentials underwent a series of changes leading to the production of giant spontaneous potentials up to 10mV's in amplitude (Usherwood, 1963b).

Rankin and Arbas (1991) found that resting membrane potential was depolarized in M#133b,c fibers following autotomy in adult grasshoppers. Additionally, they found that the size and speed of the excitatory junctional potentials was decreased. Taken together these findings suggest that muscle degeneration following autotomy may occur secondarily to direct muscle denervation. Thus, even though motoneurons maintain

terminals to their degenerating targets, these synapses may not be functional following autotomy.

Damage to multiaxonal neurons

Some thoracic muscles that degenerate following autotomy are innervated by CI motoneurons (*i.e.* M#120) and have DUM neuron innervation (Clinton and Arbas, 1995). Is it possible that the severing of one or more of these multiaxonal neurons, carries a direct signal which induces muscle atrophy after autotomy? Three facts argue against this possibility. 1) The CI does not innervate M#133b,c; the most severely affected thoracic muscle (Clinton and Arbas, 1995). 2) Backfill experiments from M#133b,c have shown that DUM neurons with axons in N3 and N4 stain in some preparations, but the stain was never seen extending to the axons running in N5 (Villalobos and Arbas, 1988). 3) Since DUM neurons have bilateral axons, one would expect any affect from axotomy to be bilateral. No atrophy on the side contralateral to autotomy has ever been seen. Therefore, the axotomy of multiaxonal neurons does not appear to be a trigger of muscle degeneration post-autotomy.

Changes in axotomized motoneurons

What effects might autotomy have on the circuitry of the metathoracic ganglion? Horridge and Burrows (1974) describe a thinning of N5 after autotomy in locust. They concluded that much of the thinning was from loss of sensory fibers, since motoneuron axons persisted in N5 one month after autotomy despite the loss of their target muscles.

In many invertebrate models, atrophied motoneuronal somata with dense perinuclear concentrations of RNA are seen soon after axotomy (Pitman *et al.*, 1972). These few gross morphological changes are unlike the significant changes seen in vertebrate motoneurons after axotomy (Sears, 1987; reviewed by Titmus and Faber, 1990).

Passive membrane properties remain stable in many different axotomized invertebrate motoneurons, while vertebrate motoneurons show great changes in passive membrane properties, which in turn affect many important neuronal characteristics (Foehring *et al.*, 1986,b; reviewed by Titmus and Faber, 1990). Axotomized invertebrate motoneurons, however, do show changes in their active membrane properties. For example, normally non-spiking somata of locust, cockroaches, and crayfish change to produce fast rising, overshooting action potentials (AP's) in response to direct current injection after axotomy or local application of colchicine (Pitman *et al.*, 1972; Goodman and Heitler, 1979; Kuwana and Wine, 1980). This suggests that newly synthesized voltage gated Na^+ and possibly K^+ channels intended for transportation into the axon are inserted into the soma and dendrites after nerve lesion. The rates of these physiological changes vary. For example, spiking somata are first seen in locust four days after axotomy, but after only 36 hours in crayfish. Some of this variation may be explained by the length of neuron left after axotomy, since the rate of muscle atrophy and the rate of change in motoneuron function after axotomy is inversely related to the length of the remaining neuronal stump (Gutmann, 1976; Kuwana and Wine, 1980). Horridge and Burrows (1974) also found that axotomized locust motoneurons lose their synaptic inputs within days. If axotomy of the motoneurons leads to the loss of interneuron input, this

might have an effect on the other interneuron targets, including the motoneurons of N3 and N4 innervating the affected thoracic muscles. A retrograde transneuronal effect in rats secondary to motoneuron axotomy has been reported by Rotshenker (1979, 1982), but in this case axotomy does not lead to remote muscle degeneration. He describes the spouting of motoneuronal terminals following the axotomy of the contralateral motoneuron. Interestingly, contralateral effects are also seen following blockade of axonal transport by colchicine. A similar transneuronal effect in invertebrates is reviewed by Nuesch (1985). When a leg nerve in the cockroach (*Periplaneta americana*) is axotomized during the 5th instar not only does the denervated muscle degenerate, but innervated muscle on the ipsilateral and contralateral sides show variable amounts of degeneration or hypertrophy. If the same lesions are make in adult cockroaches, the denervated muscle shows similar changes (myofilament loss and lipid replacement after 2 months), but no transneuronal affects are seen. The motoneurons running within N5 also could be joined by electrical junctions with the motoneurons running within N3 or N4 as is known to occur in the stomatogastric networks of crustaceans (Harris-Warrick *et al.* 1992) and during vertebrate development (Chang and Balice-Gordon, 1997). Electrical connections in grasshoppers, however, have be reported only between neurosecretory cells (Orchard and Shivers, 1986) and between two visual interneurons in the brain (Killmann and Schurmann, 1985). Thus, autotomy-induced muscle degeneration could be triggered by a retrograde signal from the axotomized motoneurons of nerve 5 to the motoneurons innervating the affected thoracic muscles.

Deafferentation

Like Horridge and Burrows, Zill *et al.* (1980) found that severed axonal stumps of sensory neurons with somata in the periphery degenerated quickly, causing deafferentation of central neurons. Deafferentation has been shown to reduce dendritic growth in developing insect nervous systems (Murphey *et al.*, 1976) and to affect cell metabolism, including decreasing protein synthesis (Meyer and Edwards, 1982). Similar changes are known to occur in vertebrates (Oswald and Rube, 1985; Deitch and Rubel, 1989a, b). In grasshoppers, deafferentation of thoracic motoneurons, induced by autotomy, would primarily occur through polysynaptic connections. Deafferentation of the motoneurons of the affected muscles would either increase or decrease motoneuronal activity depending upon whether the inputs to the motoneurons were predominantly excitatory or inhibitory. Furthermore, deafferentation may lead to a decrease in trophic support to the motoneuron (Deitch and Rubel, 1989a,b).

Changes in neuronal impulse activity have been shown to transform muscle-type (Mellon and Quigley, 1988) and change the morphology and physiology of the neuromuscular junction (Lnenicka and Atwood, 1985a, b). The effect of decreased trophic support on invertebrate motoneurons is unclear, but atrophy of mature vertebrate motoneurons following trophic loss is documented (Li *et. al.*, 1994). One possible trigger for muscle degeneration following autotomy, therefore, is deafferentation of the motoneurons innervating the affected thoracic muscles following degeneration of the sensory axons of N5. These deafferentated motoneurons may have significantly changed levels of activity, subsequently leading to muscle degeneration.

Hormonal effects on muscle

Hormones control muscle and neuronal atrophy during metamorphosis of holometabolous insects (defined as insects which have a pupal stage during metamorphosis) (Weeks and Truman, 1986a,b). The levels of ecdysteroids and juvenile hormone control metamorphosis. During metamorphosis the connections between many motoneurons and muscles must change as the animal's body shape matures. During each metamorphic molt, a wave of programmed cell death occurs, removing neurons which are not necessary for the next life stage (reviewed by Weeks and Levine, 1990). Some motoneurons, however, are maintained, innervating the same muscle throughout life (Levine and Truman, 1985), or innervating new muscle (Levine and Truman, 1985; Kent and Levine 1988).

During invertebrate and vertebrate development steroid hormones also affect muscle (Schwartz , 1992; Joubert *et al.*, 1994). Schwartz (1992) has demonstrated that, in response to the disappearance of 20-hydroxecdysone, intersegmental muscles of the tobacco hawkmoth (*Manduca sexta*) atrophy and die 30 hrs after eclosion. Intersegmental muscle death has been shown to be a consequence of PCD (Schwartz, 1992). Thus it could be that autotomy-induced atrophy is a result of some change in ecdysteroid or juvenile hormone balance.

The prothoracic glands which produce the ecdysteroids break down soon after the adult molt in grasshoppers (Chapman, 1982; Hagedorn, 1983). Ecdysteroids are also produced by the ovaries of adult female grasshoppers (Hagedorn, 1983). Since autotomy-

induced atrophy occurs in adults of both sexes and at all ages, ecdysteroids are unlikely to be involved. Some insects are able to produce small quantities of ecdysteroids from abdominal oenocytes, but oenocytes from adult locusts are unable to produce ecdysteroids (reviewed by Rees, 1985). The corpora allata, which produce juvenile hormone, however, do not degenerate during adulthood. In some insects, the flight muscles atrophy following a dispersal flight or during diapause (Nuesch, 1985). These changes have been attributed to changes in titers of juvenile hormone that occur at the same time as muscle atrophy. Usually an increase in juvenile hormone titer coincides with muscle loss, although in the Colorado potato beetle, the converse is true. Mordue (1977) has suggested that changes she observed in the locust *S. gregaria* after antennectomy were consistent with an increase in juvenile hormone titer, although she did not measure juvenile hormone levels. Her data suggest the possibility that the injury associated with autotomy leads to an increase in juvenile hormone titer, and this then leads to muscle atrophy.

Certain facts, however, argue against juvenile hormone alone as a trigger for autotomy-induced degeneration. Sham operations with significant wounding of the animal have never resulted in any muscle atrophy. All experimental results indicate that autotomy-induced degeneration is restricted to the side of autotomy. Grasshoppers, like all insects, have an open circulatory system, so any hormonal effects are likely to be bilateral. However, examples are seen where hormones effect only a small group of muscles. For example, when flight muscle degenerates other muscles are unaffected. Consequently, a complex interaction of a hormonal factor interacting with a neuronal

signal as a possible trigger of muscle degeneration following autotomy cannot be ruled-out by the above examples.

Programmed cell death as a mechanism of muscle degradation

Muscle degeneration occurs as a consequence of several of the possibilities outlined above, and in many of these cases PCD may be involved. PCD has been discussed as a mechanism for muscle or neuron degeneration after denervation (Wing, Haas and Goldberg, 1995), following the loss of trophic support during development (Martin *et al.*, 1992) and during development in the moth, *Manduca sexta* (Weeks and Truman, 1985; Fahrbach *et al.*, 1994a). Other invertebrates also demonstrate muscle atrophy as a result of PCD. *Drosophila melanogaster* exhibits nervous and muscular degeneration by PCD 12 hrs after eclosion (Kimura and Truman, 1990). In the pea aphid, *Acyrthosiphon pisum*, the indirect flight muscles degenerate by PCD following migratory flight (Kobayashi and Ishikawa, 1993). Crustaceans undergo a molt-induced atrophy of claw muscles to facilitate withdrawal of the claws at ecdysis. Shean and Mykles (1995) have demonstrated that levels of ubiquitin mRNA and ATP/ubiquitin-dependent proteasome complex increase during ecdysis, suggesting a PCD pathway. Since muscle degeneration post-autotomy is a fairly rapid process with significant changes in muscle weight occurring within nine days (Arbas and Weidner; 1991), PCD may be the mechanism of muscle degradation.

Objectives

Of the six possible triggers of muscle degeneration following autotomy, muscle disuse and axotomy of multiaxonal axons have been experimentally ruled-out. In the following chapters I will describe the time course of physiological changes in muscle following autotomy and test whether muscle degeneration is triggered by synaptic failure of the neuromuscular junction. I will determine whether the mechanism of muscle degradation following autotomy occurs via PCD. Finally, I will test whether the loss of sensory input or retrograde influences from axotomized motoneurons of N5 is a trigger for muscle degeneration, and whether juvenile hormone acts as an intermediary to muscle degeneration following autotomy.

CHAPTER TWO: MUSCLE DEGENERATION FOLLOWING REMOTE NERVE INJURY

ABSTRACT

Muscle depends upon innervation and contraction to maintain a differentiated state. Denervation can therefore induce muscle atrophy. In grasshoppers, muscle degeneration can also be triggered by the severing of a leg during autotomy. In this case, the muscles that degenerate are neither damaged nor denervated. This phenomenon suggests the existence of transneuronal mechanisms that influence muscle survival. To characterize this autotomy-induced process, we studied the degeneration of a thoracic tergotrochanteral depressor muscle (M#133b,c) subsequent to the shedding of a hind-limb in the grasshoppers, *Barytettix psolus* and *B. humphreysii*. Both histochemical and electrophysiological methods were used to follow muscle degeneration 1, 3, 5, 10 and 15 days post-autotomy. Muscle fibers began to show 'denervation-like' electrophysiological changes (*i. e.* depolarized resting membrane potentials and post-inhibitory rebound) as soon as 3 days post-autotomy. By 10 days, significant muscle degeneration was evident and electrophysiological changes were found in all animals tested. Muscle anatomical degeneration was not induced by synaptic transmission failure, because neuromuscular transmission was maintained in most fibers. The rate of muscle degeneration was not constant. Between 1 and 10 days, mean fiber cross-sectional area did not change on the autotomized side, though this is normally a time of muscle growth. However after 10 days, cross-sectional area became drastically reduced and the number of muscle fibers

within M#133b,c was decreased. The variability in rate of fiber degeneration was not dependent upon fiber type, since M#133b,c only contains 'fast-type' fibers.

INTRODUCTION

The dependence of neurons upon afferent input and feedback from their targets has been well documented during both development and maturity in vertebrates (Hubel *et al.*, 1977; Oppeinheim and Havekamp, 1988; Deitch and Rubel, 1989a,b; McMannaman *et al.*, 1990). Muscle development and maintenance has also been shown to be dependent upon neuronal input (Buller and Pope, 1977; Sanes, 1987). Invertebrate neuromuscular systems are controlled by interactions similar to those found in vertebrates (Murphey, 1986). For example, competitive interactions regulate neuron number during development; neuronal properties are dependent upon synaptic inputs (Donaldson and Josephson, 1981; Loer and Kristian, 1989); and motoneuron activity has been shown to regulate properties of the neuromuscular junction and muscle fiber phenotype in mature crabs (Mellon and Quigley, 1988; Lnenicka and Atwood, 1989). Since neuronal systems are highly dependent upon interactions with their afferents and targets, a lesion in a single neuronal component may lead to the break-down of the entire system (Le Gros Clark and Penman, 1934).

I am interested in how nerve damage may induce changes in neuromuscular systems that are far removed from the original site of injury. The grasshopper provides an advantageous system in which to study this topic because the relative simplicity of the nervous system allows the study of individual identified neurons and synaptic

connections *in vivo*. Grasshoppers have the ability to spontaneously drop a hind-limb to escape a predator. This process is called autotomy. Following autotomy, a group of thoracic muscles responsible for moving the base of the leg (coxa) degenerate, although they are neither damaged nor directly denervated. The thoracic muscles are not physically damaged during autotomy because no muscles span the plane of autotomy (McVean, 1975). Only the thoracic muscles on the side of autotomy degenerate and the hind-limb is never regenerated. The muscles are not directly denervated because autotomy severs the leg nerve (N5) while the degenerating muscles are innervated by branches of nerves 3 and 4 (figure 2.1; all nerve and muscle nomenclature according to Snodgrass, 1929).

Arbas and Weidner (1991) demonstrated that muscle degeneration is triggered transneuronally when the leg nerve is severed during autotomy. This study indicated that muscle degeneration is not secondary to leg disuse, and that hormonal interactions are unlikely. How the severing of N5 eventually affects muscle within the thorax is unknown. I have, therefore, focused on the physiological and histological changes in the neuromuscular junction and the muscle itself following autotomy in order to eventually determine the mechanisms of transneuronal muscle degeneration. For this study, I asked whether electrophysiological changes occur in one of the degenerating muscles (M#133b,c) which are similar to those usually found following denervation. If electrophysiological changes occurred, I questioned whether the affected muscle fibers would also show failed synaptic connections (*i. e.* does individual fiber denervation induce muscle degeneration) From earlier work, some fibers in M#133b,c, were known to survive up to 30 days following autotomy (Rankin and Arbas, 1991). Additionally,

'slow' and 'fast' fiber types have been shown to respond differently to both denervation and autotomy (Herbison, 1979; Clinton and Arbas, 1994). I, therefore, questioned whether the rate of M#133b,c degeneration is correlated with muscle fiber type.

This chapter reports that M#133b,c fibers show electrophysiological changes similar to those found after muscle denervation, as soon as 3 days post-autotomy. Functional denervation does not induce muscle degeneration, because fibers with abnormal physiology demonstrate intact innervation. The variability in rate of degeneration of M#133b,c is not related to fiber type, since M#133b,c is uniformly composed of 'fast'-type fibers. The overall rate of muscle degeneration is at first, gradual, but then becomes rapid between 10 and 15 days post-autotomy. Some of this work has been presented in abstract form (Personius *et al.*, 1996).

METHODS

Experimental animals

The grasshoppers were *Barytettix psolus* and *Barytettix humphreysii*, reared on a 16:8 light/dark cycle at an air temperature of 30°C. *B. humphreysii* were used after our original colony of *B. psolus* collected from central Mexico was lost to disease. Data is reported independently for both species. Animals were fed romaine lettuce with wheat germ/bran mix *ad libitum*. Radiant heat from 100W light bulbs allowed the animals to regulate their body temperatures by making positional adjustments within their cages. All experimental animals were induced to autotomize a single hind-limb on the day following

their molt to adult (day 0), by holding the grasshopper by a single leg and gently shaking. In the days following molting to adult, a grasshopper's muscles continue to grow. I, therefore, made comparisons between the autotomized and un-autotomized sides of each animal to discriminate developmental changes from those caused by autotomy.

Muscle fiber-typing and fiber cross-sectional area

Histological techniques were performed on muscle tissue 1, 3, 10 and 15 days post-autotomy of *B. psolus*. M#133b,c was removed with the cuticle at the points of origin and insertion intact. Dissections were performed in low Ca^{2+} saline to reduce muscle contraction. The muscles were placed in carboxymethylcellulose and frozen in isopentane cooled by liquid nitrogen. Only muscle samples in which the frozen lengths were the same as the *in situ* muscle length were further processed. Preparations were mounted with Tissue TEK on the cold chuck (-20°C) of a cryotome (B/K Instruments) and serial cross sections (20μm) were placed on dry, glycerin-subbed cover slips.

Measurement of fiber myosin ATPase (mATPase) activity was performed at a pH of 9.4 as described by Muller *et al.* (1992). To detect possible isoforms of mATPase on the basis of different pH stabilities, sections were pre-incubated for 30 minutes in solutions of pH 4.5 or pH 10.4 (n = 3-5). M#120 which contains both 'fast' and 'slow' fibers was included in all experimental runs as a control of staining intensity. Muscle fiber typing was verified by succinic acid dehydrogenase (SDH) activity according to the

method of Nolte and Pette (1972). As a negative control, succinate was not added to the staining medium (n = 3).

The extent of muscle atrophy was determined by measuring the cross-sectional area of all fibers at about the midpoint of the muscles. To assess for possible fiber shrinkage during the mATPase protocol, we compared the cross-sectional areas of adjacent serial sections stained for mATPase with sections fixed in 4% formaldehyde and 1% glutaraldehyde and then stained with hematoxylin and eosin (H&E). Sections stained for mATPase or H&E showed no difference in fiber cross-sectional area. Both mATPase and H&E stained samples were used to determine cross-sectional area. Cross-sectional area was determined from camera lucida tracings with a morphometric program (SigmaScan).

Electrophysiology

Electrophysiological recordings were made 1, 3, 5, 10 and 15 days post-autotomy in *B. psolus* and 3, 10 and 15 days post-autotomy in *B. humphreysii*. The thoracic cavity of experimental animals was opened by dorsal and ventral mid-line cuts and both thoracic halves were pinned out. The contralateral un-autotomized M#133b,c served as an internal-control in all electrophysiological experiments. M#133b,c was exposed with both origin and insertion intact. All preparations were continuously superfused with grasshopper saline containing (in mM) 150 NaCl, 5 KCl, 5 $CaCl_2$, 1 $MgCl_2$, 90 sucrose, 5 trehalose and 5 Tris maleate at pH 7.2. The branch of nerve 3 innervating M#133b,c was

severed and a suction electrode attached for stimulation experiments. Intracellular recordings from muscle fibers were made in current-clamp mode (Axoclamp 2A, Axon Instruments) with microelectrodes (20-40 MΩ) filled with 3M K acetate. Recordings were made from up to 20 fibers per muscle, approximately 1/3 of the total number of fibers within M#133b,c (n = 3-6 animals per species for each time post-autotomy).

Resting membrane potential (RMP): RMP was determined by the voltage difference between the inside of a muscle fiber and ground.

Spontaneous miniature excitatory junctional potentials (mEJPs): mEJPs were identified as approximately 0.5 mV deflections of fast onset. The voltage deflections were verified as mEJPs by replacing the bath solution with low Ca^{2+}/ high Mg^{2+} saline, which reduces synaptic transmission, but has no affect on the mEJPs (Fatt and Katz, 1952). The percentage of fibers displaying mEJPs was determined for all experimental animals. In five animals 10 days post-autotomy, mEJP size and frequency over a one minute duration was determined for 30 muscle fibers.

Post-inhibitory rebound: Post-inhibitory rebound was identified by an overshooting voltage spike after the muscle fiber was strongly hyperpolarized. Normal fibers did not demonstrate post-inhibitory rebound even after they were induced to depolarize by a second current-passing electrode (Marshall and Ward, 1973).

Evoked excitatory junctional potentials (eEJPs): To determined whether muscle fibers displaying post-inhibitory rebound or low RMP's maintained a functional neuromuscular junction, eEJPs were evoked via a suction electrode on nerve 3 in 5 animals 10 days post-autotomy (34 muscle fibers). Stimulus duration ranged from 0.5 to

1.0 milliseconds. Muscle contraction was blocked by 0.6-1M formamide in grasshopper saline (Cordoba *et al.*, 1968).

Wet weight: Muscle wet weight was determined after each electrophysiological experiment. Each muscle was blotted on filter paper to remove surface moisture and weighed with accuracy of $\pm$ 0.01 mg (n = 3-6 animals per species for each time post-autotomy).

Statistics

All data were first assessed for normality. A single-tailed paired *t*-test was used to compare autotomized vs. un-autotomized muscle weights and mEJP frequency. Percentage data for post-inhibitory rebound and mEJP was normalized by arcsine transformation and a two tailed paired t-test performed. A one-tailed un-paired t-test was used to compare autotomized vs internal-control or internal-control vs un-autotomized muscle fiber cross-sectional area. RMP data was not normally distributed and a Kruskal-Wallis one way ANOVA with Mann-Whitney post-hoc test was used. Significance was set to $p \leq 0.05$ (Sigmastat or Statmost computer programs).

RESULTS

Rate of muscle fiber degeneration:

When muscle wet weight was measured following autotomy, *B. psolus* and *B.*

humphreysii showed a similar rate of muscle growth on the internal-control side and muscle degeneration on the side of autotomy (figure 2.2). Muscle wet weight increased on the internal-control side from day 1 to day 15. The growth rate of M#133b,c was similar to the rate found by Arbas and Weidner (1991) for a group of six metathoracic-coxal muscles following the final molt. Muscle mass on the autotomized side declined following autotomy and was significantly less than internal-control on day 5 in *B. psolus*, and on day 10 and day 15 in both species (muscle weight was not determined on day 5 in *B. humphreysii*).

To further characterize the rate of muscle degeneration subsequent to autotomy, I determined the change in average fiber cross-sectional area (CSA) following autotomy in *B. psolus*. Average fiber CSA increased on the internal-control side as the animal matured (~30 μm^2 one day after autotomy to ~100 μm^2 by 10 days). After 10 days, average fiber CSA on the internal-control side was stable (figure 2.3b). Little or no growth occurred in the muscle fibers on the autotomized side, shown by the average fiber CSA remaining constant until day 10, although some variability in fiber size was found (figure 2.3b). By 15 days, however, the average fiber CSA was greatly decreased (11.0 ± 9.0 μm^2).

M#133b,c generally contained ~60 fibers. At first, M#133b,c on the autotomized side and the internal-control side contained the same number of fibers (1 day 56.4 ± 6.2 vs. 59.8 ± 2.3; 3 days 58.0 ± 14.4 vs. 59.7 ± 9.1; internal-control vs. autotomized side: n = 3-4), but by 10 days fiber number on the autotomized side was greatly reduced in some

animals (3) and unchanged in others (2). The fiber number was markedly reduced in all animals on the side of autotomy after 15 days (66.4 ± 6.6 vs. 10 ± 6.3; figure 2.3a). Unsurprisingly, total fiber CSA, which is dependent upon average fiber CSA and fiber number, declined on the side of autotomy in a similar manner as wet muscle weight. A significant difference in total fiber CSA was found between the experimental and control sides 3 days post-autotomy. Autotomy did not cause hypertrophy of M#133b,c in the remaining limb since muscle wet weight, average fiber cross-sectional area, and summed cross-sectional area of normal un-autotomized animals did not differ from the internal-control side of autotomized animals (figure 2.2; figure 2.3b and c). Figure 2.4 demonstrates the extent of muscle degeneration 15 days after autotomy. Note that some of the very small remaining fibers no longer stain for myosin ATPase (arrow).

Muscle #133b,c composition:

The variability in the rate of muscle fiber degeneration was not related to differences in fiber-type since, M#133b,c contained only 'fast-type' fibers. The high intensity of myosin ATPase staining at pH of 9.4 and after preincubation at pH 4.5, with an absence of staining after preincubation at pH 10.4, indicated that all the fibers were of the 'fast' type (figure 2.5a,b; Muller *et* al., 1992). M#120 which contains both 'fast' and 'slow' fibers was included in all experiments as a control for staining intensity (figure 2.5d, e). Staining for SDH was performed to verify fiber-typing results. M#133b,c was stained only lightly for SDH, an oxidative enzyme commonly found in lower

concentrations in 'fast' fibers than 'slow' fibers (Nolte and Pete, 1972; figure 2.5c). *B. psolus* were used in M#133b,c fiber type experiments.

Muscle fibers remain innervated during the degeneration process:

Invertebrate neuromuscular junctions show three types of eEJPs: fast eEJPs which overshoot zero, fast eEJPs and slow eEJPs depending upon motoneuron type and muscle membrane properties. Examples of these eEJP types from *B. humphreysii* are shown from M#133b,c in the top panel of figure 2.6b. Neuromuscular transmission was always maintained 10 days post-autotomy when the muscle fiber RMP was above -30 mV, indicating that the neuromuscular junction was still functional in abnormal fibers (figure 6a). When muscle fiber RMP fell below -30 mV, however, the percentage of fibers with functional neuromuscular junctions decreased. Evoked EJPs were, however, seen in fibers with RMP's as low as -13 mV (figure 2.6b). The size of the evoked EJPs was usually maintained on the side of autotomy until the muscle fiber RMP depolarized beyond -50 mV's (figure 2.6c). The eEJP time to peak (time from stimulus to eEJP peak) was not dependent upon muscle fiber RMP, but when RMP on the side of autotomy was depolarized to less than -60 mV, the time to peak was abnormally long in some fibers (figure 2.6d). Muscle fibers on the autotomized side also frequently demonstrated slow eEJPs (figure 2.6d). Slow eEJPs were rarely demonstrated in internal-control fibers, perhaps because they were obscured by the larger fast eEJPs.

Electrophysiological changes in M#133b,c during muscle fiber degeneration:

Median RMP of M#133b,c on the internal-control side was generally maintained between -60 and -75 mV (25th and 75th percentiles of all internal-control data) and, did not change as the animal matured (figure 2.7). The RMP was lower on the autotomized side of all animals, but the difference between the two sides was significant in only a single animal 1 and 5 days after autotomy, and in three of nine animals 3 days post-autotomy. By 10 days post-autotomy, all animals demonstrated significantly depolarized muscle fibers on the autotomized side. Fifteen days post-autotomy, stable recordings could be made from only a few muscle fibers (avg 13 per muscle) and, only six of nine animals demonstrated significantly depolarized muscle fibers on the side of autotomy (figure 2.7).

Beginning 3 days post-autotomy, muscle fibers demonstrated increased membrane excitability. Nearly 30% of muscle fibers on the autotomized side demonstrated post-inhibitory rebound 3 days post-autotomy compared to 3% of contralateral-control fibers (figure 2.8a). Five days post-autotomy nearly 50% of fibers showed post-inhibitory rebound. By 10 days post-autotomy, the percentage was increased to 65% and fibers often showed multiple spikes after hyperpolarization (figure 2.8b). Only 15% of muscle fibers continued to show post-inhibitory rebound 15 days post-autotomy (figure 2.8a). Post-inhibitory rebound was not dependent upon muscle fiber RMP. Despite a wide range in normal RMP, control muscle fibers rarely showed post-inhibitory rebound (5 / 520 fibers). Post-inhibitory rebound never occurred when internal-control fibers were depolarized by positive current injection from one microelectrode and briefly hyperpolarized by a second electrode (Marshall and Ward, 1974; data not shown).

Muscle fibers on the autotomized side also showed changes in spontaneous mEJPs. The percentage of muscle fibers showing visible mEJP's was increased by 1 day post-autotomy (70% compared to 55%), but there was great variation between animals. The difference persisted, until 15 days post-autotomy when the autotomized side was similar to the contralateral-control side (38%), but was only significant at 10 days post-autotomy (60% to 38%). The change in percentage of fibers showing mEJPs could be caused either by a pre-synaptic or by a post-synaptic change in the neuromuscular junction. We, therefore, determined the change in mEJP frequency after autotomy. Using the same muscle fibers as those in our experiments of eEJP, we found that the frequency of mEJPs in these fibers 10 days post-autotomy was increased in muscle fibers on the side of autotomy (figure 2.9a). There was no difference in the average size or variance of mEJPs in internal-control fibers and fibers on the side of autotomy (figure 2.9b). Example mEJPs are shown in figure 2.9c.

DISCUSSION

I have shown that a mature muscle, though it still has a functional neuronal innervation, may be induced to degenerate following the severing of a synaptically remote leg nerve during autotomy. Electrophysiological differences between the homologous muscle on the two sides of the insect were observed in some animals only 3 days after autotomy (figure 2.6 and 2.8). By 10 days, all animals demonstrated a reduction in RMP on the side of autotomy and post-inhibitory rebound was evident in most muscle fibers.

At 15 days post-autotomy, however, the results for median fiber RMP, percentage of fibers showing post-inhibitory rebound and/or mEJPs returned towards control values in some animals (figures 2.6 and 2.8). There are three possible explanations for this occurrence. One, some fibers may be resistant to degeneration. Two, individual muscle fibers may recover following autotomy. Three, all fibers may degenerate but at different rates such that 15 days post-autotomy only the larger fibers could be penetrated with a microelectrode. Since average CSA was significantly reduced in all muscle fibers 15 days post-autotomy, no muscle fibers appear totally resistant to degeneration. Moreover, sections of M#133b,c taken from animals 30 and 60 days post-autotomy never showed recovery of fiber cross-sectional area, so individual fibers do not regenerate after initially atrophying (Rankin and Arbas, 1991). The apparent return of muscle fiber electrophysiological properties toward control values appears to be an artifact due to sampling. By 15 days post-autotomy, stable intracellular recordings could probably be made only from the largest fibers, the slowest to degenerate. As the slowest to degenerate, they would have had relatively normal electrophysiological properties.

Muscle #133b,c undergoes electrophysiological changes that resemble those found in muscle after denervation. Denervated vertebrate muscle fibers show a range of changes in their electrophysiological properties. Classical findings include: a reduced RMP (Redfern and Thesleff, 1971); an increased extrajunctional sensitivity to neurotransmitter, acetylcholine (Axelsson and Thesloff, 1959); muscle fibrillation starting from changes in spontaneous miniature membrane potential changes (Thesleff and Ward, 1975); and the presence of post-inhibitory rebound (Marshall and Ward, 1974).

Invertebrate muscle fibers show similar changes after denervation. Invertebrate changes include: a reduced RMP (Usherwood, 1963a); an increased extrajunctional sensitivity to neurotransmitter, glutamate (Gration *et al.*, 1978); and changes in the size and frequency of spontaneous miniature membrane potentials (Usherwood, 1963b). We found many of the same changes in muscle fiber electrophysiology, and these 'denervation-like' changes occur irrespective of the animal's age, since similar electrophysiological changes also occur in sexually mature grasshoppers (Rankin and Arbas, 1991).

This 'denervation-like' muscle degeneration was not due to individual muscle fiber denervation, since the neuromuscular junctions were found to be functional in many abnormal fibers 10 days following autotomy. Some fibers, though, demonstrated a reduction eEJP size. This may simply reflect the decreased average RMP of autotomized muscle fibers, but eEJP size was sometimes abnormally small even when muscle fibers were only slightly more depolarized than internal-control fibers. The decreased eEJP size was, therefore, probably due to pre-synaptic weakening of the neuromuscular junction.

mEJP size is dependent upon mEJP current and fiber input resistance. Input resistance is a function of both membrane resistance and fiber diameter. When fiber diameter decreases during muscle degeneration, fiber input resistance should increase, increasing the mEJP size. The average mEJP size and variability of mEJPs size, however, did not change following autotomy. Thus, membrane resistance probably decreases by an amount that leaves fiber input resistance unchanged, assuming quantal size (mEJP current) does not change. mEJPs as small as 0.15 mV were detectable by the characteristic small rapid voltage change and baseline defection caused by mEJPs.

Smaller mEJP's would have been lost in the noise of our traces (~ 0.10 mV).

An increase in mEJP frequency may be caused by a pre-synaptic change or an increase in the post-synaptic cell's input resistance. The increased input resistance would increase mEJP size and, therefore, the number of detectable mEJPs. We found that mEJP size did not change following autotomy, so mEJP frequency changes cannot be explained by an increased input resistance. An increased fiber length constant may also increase the number of detectable mEJPs, by increasing the chance of seeing mEJPs from distant synaptic arbors. However, as the muscle fiber diameter decreases following autotomy, the length constant would be expected to decrease, lessening the likelihood of seeing mEJPs from distant sites. I conclude, therefore, that the changes found at the neuromuscular junction appear to be from presynaptic changes. The longer eEJP latency could also be caused by a pre-synaptic mechanism (*e.g.* slowed axon conduction velocity).

The rate of degeneration of M#133b,c was widely variable, both between and within animals, even though it was composed entirely of 'fast' muscle fibers. Following autotomy, the fibers on the side of autotomy failed to increase in size, and were significantly reduced in size by day 15. Changes in fiber number were detectable somewhat earlier (10 days; figure 2.3a). The results seem to suggest that degeneration of the muscle fibers occurred rapidly since, until day 15, few small fibers were found on the autotomized side, despite the reduction in fiber number by 10 days. Although the fibers on the autotomized side did not grow, they continued to stain for myosin ATPase until they disappeared. I have recently found that many M#133b,c fibers label positively for an

ubiquitin-antibody 15 days post-autotomy (Personius and Chapman, 1997). Ubiquitin is a highly conserved 76-amino acid protein that is expressed in all eukaryotes (Rechsteiner, 1988). Following covalent attachment of ubiquitin molecules to a protein, the protein is rapidly lyzed by an ATP-dependent protease complex (Eytan *et al.*, 1989). Ubiquitin is expressed in increased amounts in some tissues undergoing programmed cell death (*e.g.* larval muscle in some moths following ecdysis; Farhbach and Schwartz, 1994). I have found that those fibers which show ubiquitin-like immunoreactivity frequency stain lightly for myosin ATPase. This may explain why fiber degeneration seems to be so rapid. Once ubiquitin is expressed, the muscle fiber is rapidly degenerated leaving the very small fibers seen in figure 4 which are nearly devoid of contractile apparatus.

How these changes occurred in an innervated muscle fiber is an interesting question. The motoneurons running within N5 are known to survive following axotomy, but since the cell bodies of N5 sensory afferents are located in the periphery, their axon terminals within the central nervous system degenerate (Horridge and Burrows, 1974). The degenerating afferent axons are thought to interact predominately with the motoneurons of nerves 3 and 4 via poly-synaptic connections within the metathoracic ganglion (Burrows 1992; Laurent 1987). Thus, the severing of N5 may lead to trans-neuronal orthograde deafferentation of the nerve 3 and 4 motoneurons and a quiescence neuromuscular junction. The silent junction may lead to a 'denervation-like' muscle degeneration.

Figure 2.1 Schematic drawing of a grasshopper metathoracic ganglion and possible neuronal interaction affected by autotomy. Autotomy severs the leg nerve (N5) between the leg trochanter and femur. N5 carries motoneurons innervating distal leg musculature and leg afferents. These multiple leg afferents mainly terminate on metathoracic interneurons (open circles). A few leg afferents may synapse directly on motoneurons. The thoracic muscles which degenerate are innervated by motoneurons (filled circles) within nerves three and four (N3 and N4).

Figure 2.1

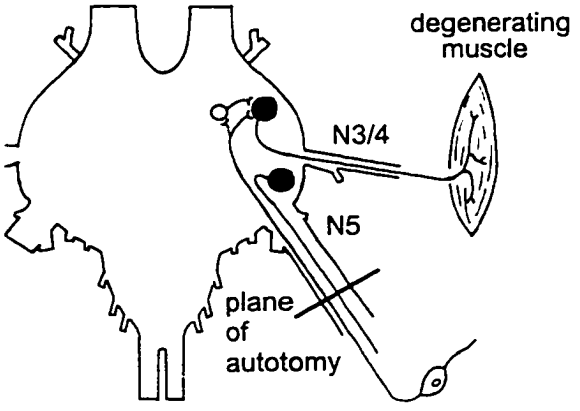


Figure 2.2 Muscle #133b,c wet weight: triangle- un-autotomized animals (right and left sides); circle- *B. psolus* (filled circle, autotomized side; open circle, internal-control side); square- *B. humphreysii* (filled square, autotomized side, open square, internal-control side). *B. psolus* and *B. humphreysii* show the same rate of muscle growth on the internal control side and muscle degeneration on the autotomized side. No differences are seen between the un-autotomized animals and the internal-control side of either species. Differences in weight occur between the autotomized and internal-control sides in *B. psolus* at 5 days and both species at 10 and 15 days (one-way paired t-test; n = 3-6 animals; * $p < 0.05$).

Figure 2.2

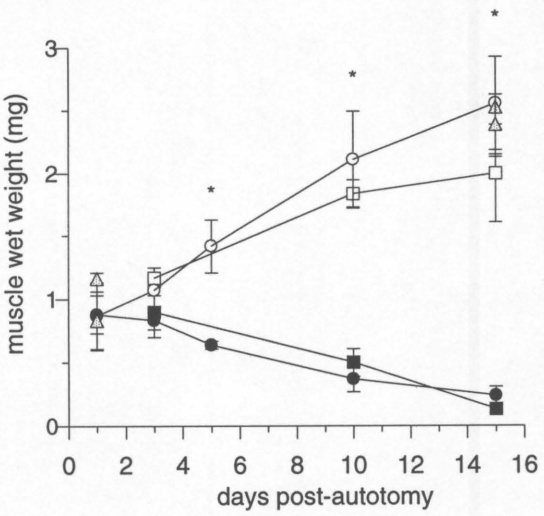

muscle wet weight (mg)
0
1
2
3
0
2
4
6
8
10
12
14
16
days post-autotomy
*
*
*

Figure 2.3 Changes in muscle #133b,c fiber number, average fiber cross-sectional area (CSA) and total cross-sectional area (μm^2) following autotomy (*B. psolus*, mean and SE; n = 3-5); triangle- un-autotomized animals; filled circle- autotomized side; open circle- internal-control side. A. Muscle #133b,c generally contains 60 fibers. Muscle fiber number is decreased in some animals 10 days post-autotomy, and decreased in all animals 15 days post-autotomy. B. Average fiber cross-sectional area; significant differences are seen between the autotomized and the internal control sides 10 and 15 days post autotomy. No difference is seen between the internal-control side and un-autotomized animals. C. Total cross-sectional area; significant differences are seen between the autotomized and internal-control sides 3, 10 and 15 days post-autotomy. No difference is seen between the internal-control side and un-autotomized animals (all comparisons by one-way unpaired t-test; * $p < 0.05$).

Figure 2.3

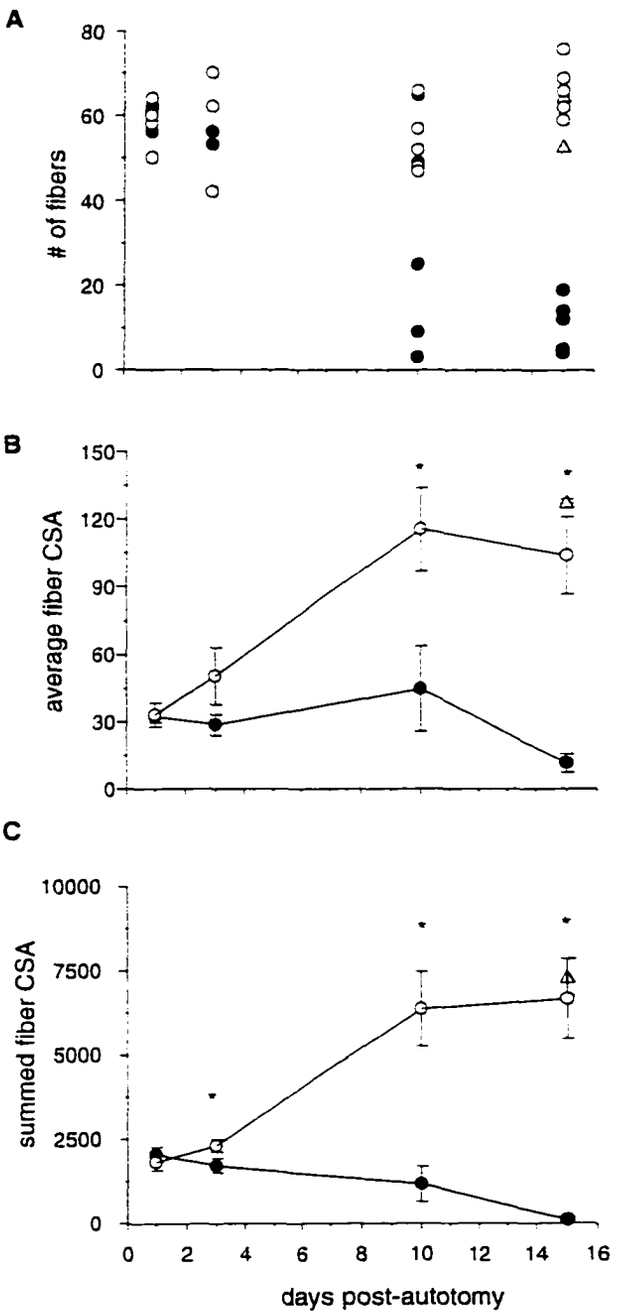

A
# of fibers
80
60
40
20
0
B
average fiber CSA
150
120
90
60
30
0
C
summed fiber CSA
10000
7500
5000
2500
0
0
2
4
6
8
10
12
14
16
days post-autotomy

Figure 2.4 Myosin ATPase staining (pH 9.4) of muscle #133b,c 15 days post-autotomy. A. Part of an internal-control muscle. B. Whole muscle on the autotomized side of the same animal; note only a few atrophied fibers remain. Arrow indicates small round fibers which stain lightly for myosin ATPase. Scale bar = 25μm.

Figure 2.4

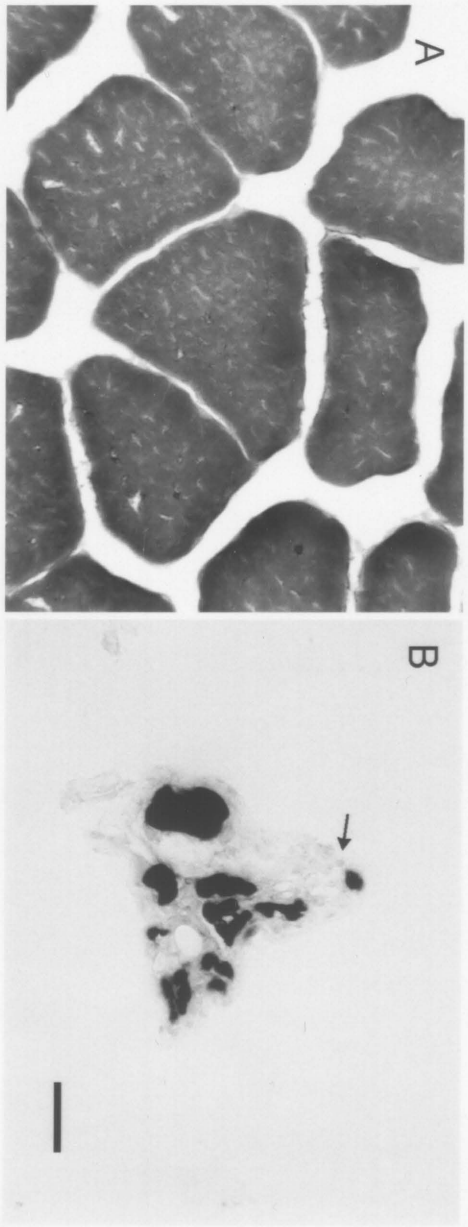


Figure 2.5 Histochemistry of muscle #133b,c (A-C) and muscle #120 (D-E) from *B. psolus*. Muscle #120 served as a control for staining techniques. A. Overall myosin ATPase activity (pH 9.4); 'fast' fibers stain dark, if present 'slow' fibers would stain light. B. pH stability of myosin ATPase was tested after alkaline preincubation at 10.4, 'fast' fibers stain light, if present 'slow' fibers would stain dark. C. M#133b,c stains lightly for succinic acid dehydrogenase (SDH). D. Myosin ATPase activity (pH 9.4); 'fast' fibers are dark, 'slow' fibers are light. E. Myosin ATPase activity (preincubation at pH 10.4); 'fast' fibers are light, 'slow' fibers are dark. Scale bar = 100μm.

Figure 2.5

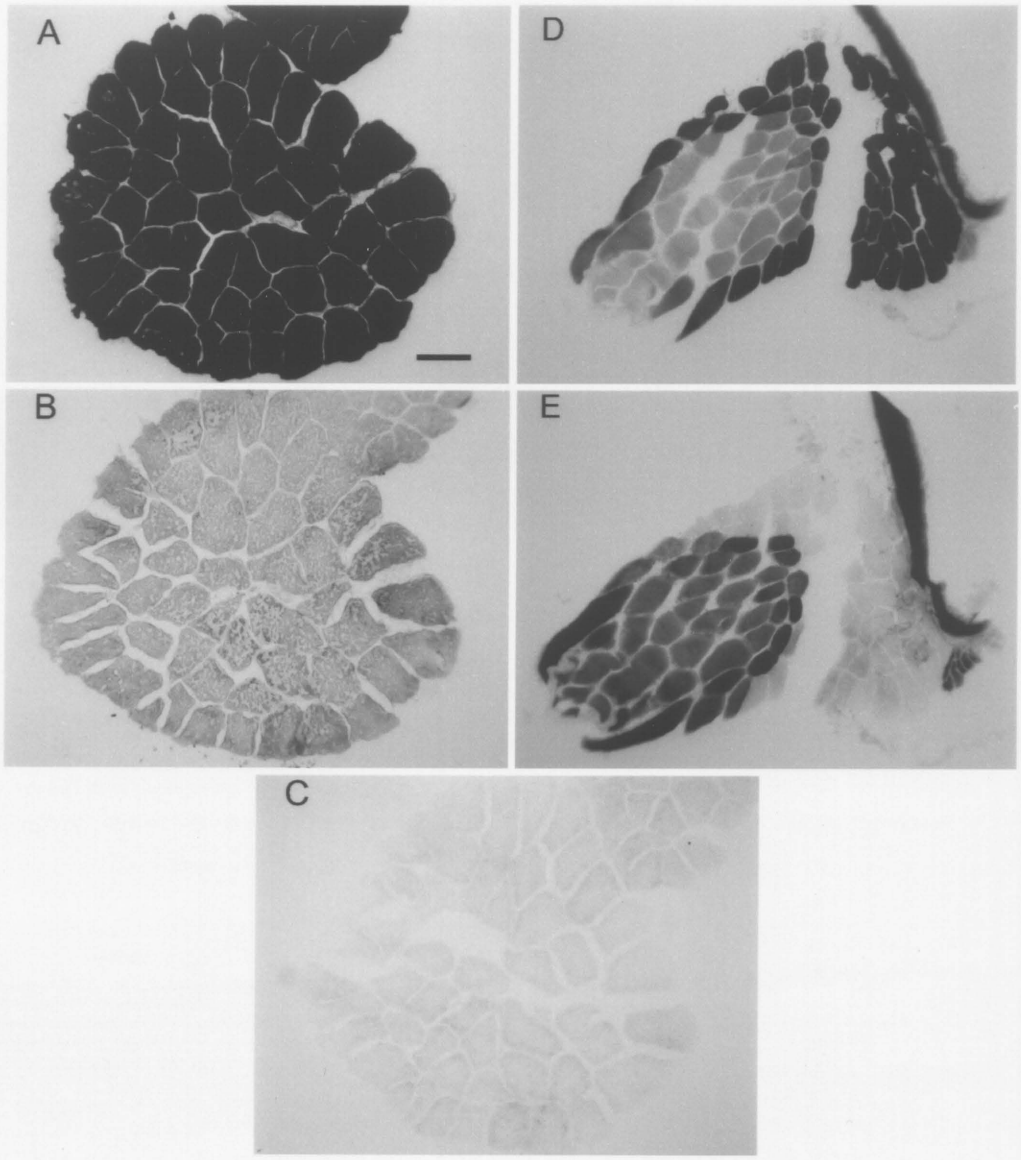


Figure 2.6 Changes in the neuromuscular junction 10 days post-autotomy. A. When muscle fiber resting membrane potential (RMP) ranges between -30 and -70 mV, evoked excitatory junctional potentials (eEJPs) are always present (numbers above bars are the number of fibers per bin). B. Example eEJPs from internal-control fibers (eEJPs which overshoot zero, fast and slow eEJPs, respectively). The fibers from the autotomized side generally showed smaller eEJPs (fast, slow and very small eEJPs, respectively). Numbers below each record are the fiber's RMP. C. Average 'fast' eEJP amplitude on the internal-control side is slightly decreased in depolarized muscle fibers (open circles). The average 'fast' eEJP amplitude on the side of autotomy (filled circles) is similar to the internal-control side, until RMP is below -50 mV when eEJP amplitude drastically decreases. Internal-control fibers rarely slowed slow eEJP's. Some muscle fibers on the side of autotomy showed both slow and fast eEJP's. Slow eEJP size did not vary with RMP (filled triangles). D. eEJP time to peak (time from end of stimulus to eEJP peak) does not vary with RMP. When RMP was more depolarized than -60 mV, eEJP time to peak is longer than normal.

Figure 2.6

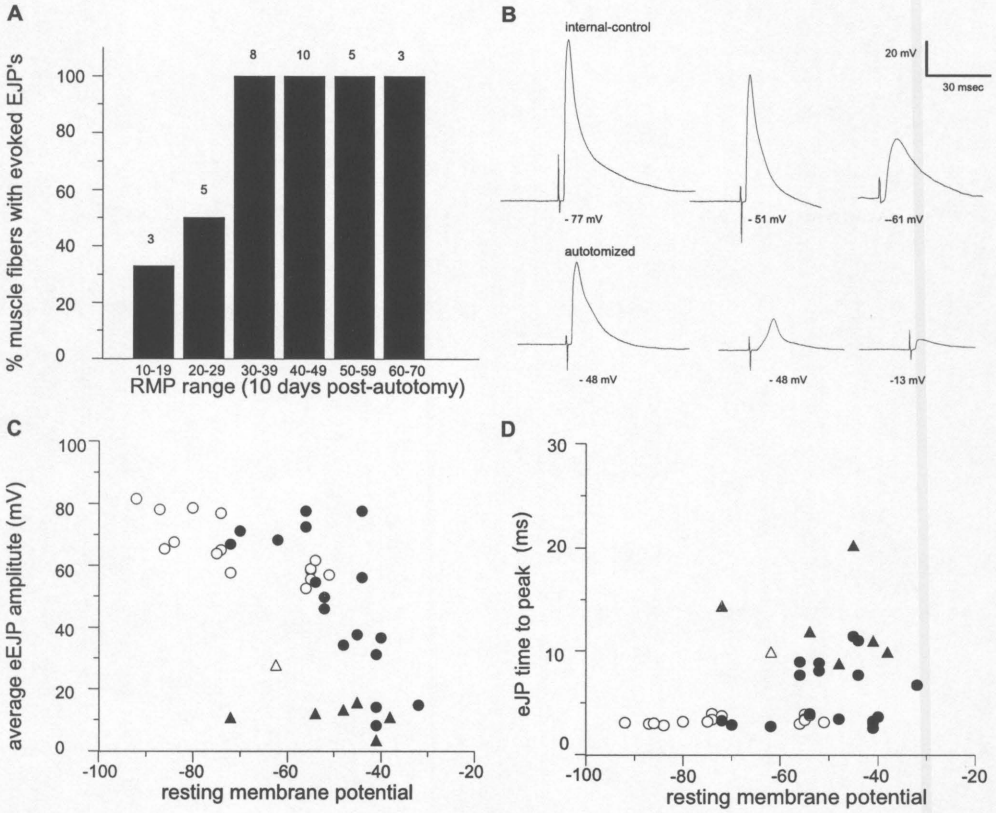

A
% muscle fibers with evoked EJP's
100
80
60
40
20
0
3
5
8
10
5
3
10-19
20-29
30-39
40-49
50-59
60-70
RMP range (10 days post-autotomy)
B
internal-control
20 mV
30 msec
- 77 mV
- 51 mV
–61 mV
autotomized
- 48 mV
- 48 mV
-13 mV
C
average eEJP amplitute (mV)
100
80
60
40
20
0
-100
-80
-60
-40
-20
resting membrane potential
D
eJP time to peak (ms)
30
20
10
0
-100
-80
-60
-40
-20
resting membrane potential

Figure 2.7 Muscle fiber resting membrane potential (RMP) is depolarized on the side of autotomy (median value for each insect). Triangle- un-autotomized animals (right and left sides); circle- *B. psolus* (open circle, internal-control; filled circle, autotomized side); square- *B. humphreysii* (open square, internal-control; filled square, autotomized side). The horizontal lines represent the 25th and 75th percentile of median RMP on the internal-control side for all animals. Median RMP does not change on the internal-control side as the animal matures. The average RMP is lower on the autotomized side in some animals 1, 3, 5 and 15 days post-autotomy, but at 10 days post-autotomy all animals demonstrate significantly depolarized muscle fibers on the side of autotomy. All symbols between the brackets represent animals whose autotomized sides are significantly different from internal-control (Kruskal-Wallis one way ANOVA with Mann-Whitney post-hoc test; * $p < 0.05$). [1] The animal with the lowest median RMP did not show a significant difference between the two sides, because only four fibers could be penetrated on the autotomized side of this animal, which reduced the power of the Mann-Whitney post-hoc test.

Figure 2.7

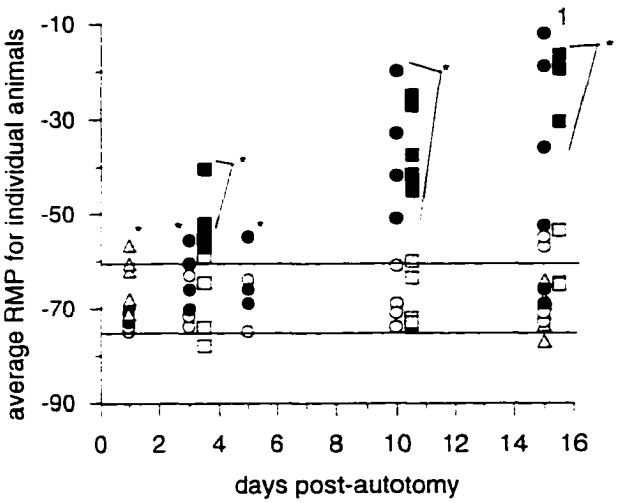

average RMP for individual animals
-10
-30
-50
-70
-90
0
2
4
6
8
10
12
14
16
days post-autotomy
1

Figure 2.8 Percentage of muscle #133b,c fibers showing post-inhibitory rebound. A. A significant increase in the number of fibers displaying post-inhibitory rebound was seen 5 and 10 days following autotomy (two tailed paired t-test following normalization by arcsine transformation; * $p < 0.05$). B. Examples of post-inhibitory rebound from an animal 10 days post-autotomy (arrow indicates start of hyperpolarization). The resting potential of the muscle fiber was -68 mV's, well within the normal resting membrane potential range (-60 to -75 mV's). Formamide was added to the saline to prevent muscle contraction.

Figure 2.8

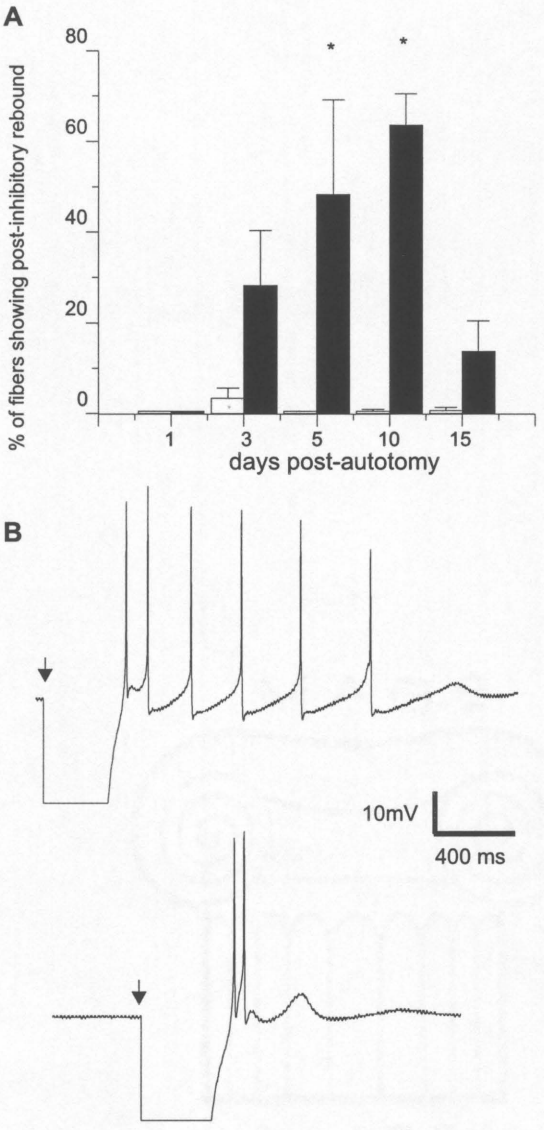
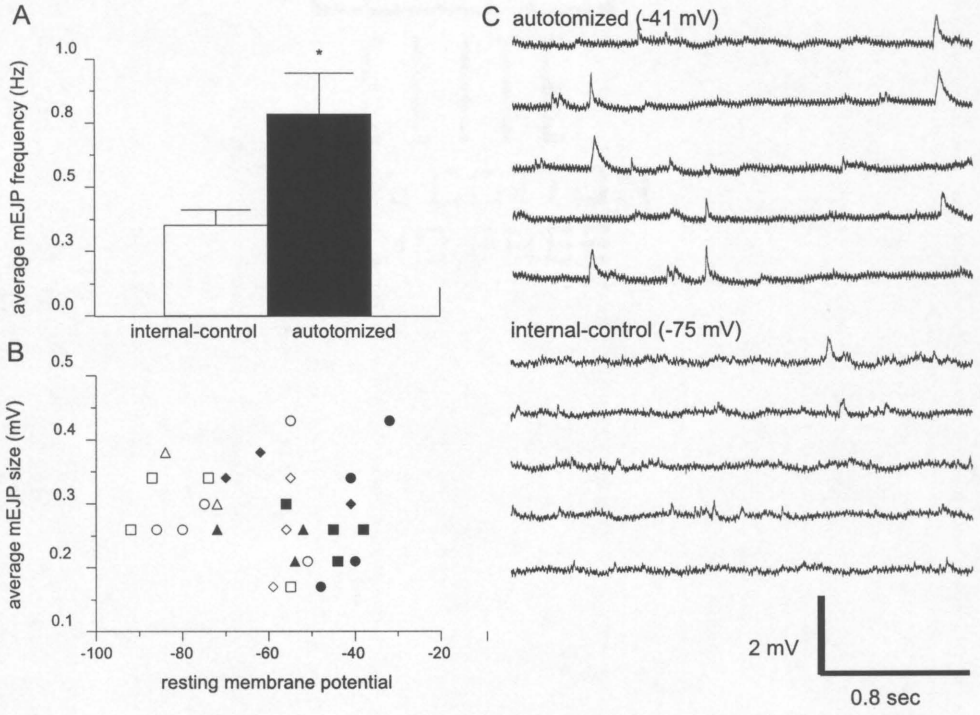

A
80
60
40
20
0
% of fibers showing post-inhibitory rebound
*
*
1
3
5
10
15
days post-autotomy
B
10mV
400 ms

Figure 2.9 Changes in spontaneous miniature excitatory junctional potentials (mEJP's) 10 days post-autotomy. A. The average frequency of mEJP's is increased on the side of autotomy compared to internal-controls (one-way paired t-test; $n = 4$ animals; * $p < 0.05$). B. There is no difference in mEJP size between the internal-control and autotomized sides (each symbol is an individual animal; open symbol-internal-control, filled symbol-autotomized side). **C.** Sample traces from fibers on the internal-control and autotomized sides of an animal.

Figure 2.9


A
average mEJP frequency (Hz)
1.0
0.8
0.5
0.3
0.0
*
internal-control
autotomized
B
average mEJP size (mV)
0.5
0.4
0.3
0.2
0.1
-100
-80
-60
-40
-20
resting membrane potential
C
autotomized (-41 mV)
internal-control (-75 mV)
2 mV
0.8 sec

CHAPTER THREE: PROGRAMMED CELL DEATH IN A MUSCLE FOLLOWING REMOTE NERVE INJURY

ABSTRACT

When the grasshopper, *Barrytettix humphreysii*, sheds a hindlimb during an escape response called autotomy, certain thoracic muscles degenerate although they are neither damaged nor denervated. Degeneration of one of these thoracic muscles, tergotrochanteral muscle #133b,c (M#133b,c), is generally complete by 15 days post-autotomy, and occurs by a two step process (Personius and Arbas, 1998). The later phase, during which both fiber size and fiber number are significantly reduced, has here been shown to occur via programmed cell death (PCD). Indicators of PCD including, increased ubiquitin-immunoreactivity, chromatin consolidation and positive terminal deoxynucleotidyl transferase mediated dUTP nicked end labeling (TUNEL), are all present 10 and 15 days following autotomy. Furthermore, the rate of muscle degeneration can be slowed by cycloheximide, a translational blocker. Muscle fibers which contain increased ubiquitin-immunoreactivity also demonstrate decreased labeling for filamentous actin. The onset of PCD is variable between individual muscle fibers. Some fibers show increased ubiquitin-immunoreactivity as early as 3 days following autotomy, while other fibers appear intact 15 days post-autotomy. This suggests the trigger for PCD is fiber specific and is not due to a general signal to the entire muscle.

INTRODUCTION

Muscle degeneration occurs as part of the normal developmental process in many insects. For example in Lepidoptera and Diptera, abdominal muscles required by the animals to escape from their pupal cuticle degenerate immediately following adult eclosion (Lockshin, 1981; Schwartz and Truman, 1982; Schwartz, 1986 and Kimura and Truman, 1990), while other insects show degeneration of indirect-flight muscle following a dispersal flight (Nuesch, 1985; Kobayashi and Ishikawa, 1994). In several of these cases degeneration is known to be a consequence of programmed cell death (PCD). Lockshin and Williams introduced the term PCD in a series of papers describing the breakdown of intersegmental muscles after eclosion in the silkmoth, *Antheraea polyphemus* (1964; 1965a-c). The term 'programmed' was used because muscle degeneration was part of an orchestrated change in the animal's morphology during development; furthermore, *de novo* protein synthesis was required for the process to occur (Lockshin, 1981).

Muscle degeneration, which occurs as a consequence of PCD, during insect development is often a two step process. In the hawkmoth, *Manduca sexta*, and the silk moth, *A. polyphemus*, the first phase of slower degeneration is triggered by a decline in the hormone 20-hydroxydecdysone (20-HE). The second more rapid phase, where muscle fibers depolarize and then fail to contract, is triggered by a further decline of 20-HE in *M. sexta* and by release of eclosion hormone in *A polyhemus* (Schwartz, 1986). Muscle degeneration by PCD is rapid process, occurring 30-48 hours after eclosion in

both species. Degeneration of abdominal muscles in *Drosophila melanogaster* abdominal muscle degeneration follows basically a similar time course (Kimura and Truman, 1990).

In the adult pea aphid, *Acyrthosiphon pisum*, muscles degenerate by PCD following migration. Muscle degeneration in the pea aphid is slower than in the insects discussed previously, occurring over a period of 7 days. Degeneration is thought to occur via PCD because it can be blocked by inhibitors of protein or RNA synthesis and a set of specific proteins is synthesized following flight (Kobayashi and Ishikawa, 1994). The trigger for muscle degeneration has been shown to be associated with feeding upon host plants after dispersal flight (Kobayashi and Ishikawa, 1993), which may lead to a rise in juvenile hormone titer (Rankin and Riddiford, 1977).

Apoptosis is a very common form of PCD which has been described for many cell types (Fraser *et al.*, 1996). Kerr *et al.* (1972) first observed that the ultrastructural changes in most mammalian cells undergoing PCD were similar. These changes include membrane blebbing, chromatin condensation and DNA fragmentation. Although degenerating mammalian cells and the abdominal muscles of *M. sexta* both require *de novo* gene expression, the same death processes are not involved, since during *M. sexta* muscle degeneration neither membrane blebbing nor DNA fragmentation occurs (Schwartz *et al.*, 1993). Furthermore, the abdominal muscles of *M. sexta* show a dramatic increase in expression of ubiquitin, a marker which tags proteins for destruction by an ATP-dependent protease complex (Eytan *et al.*, 1989), and one of the first genes shown to be up-regulated during PCD (Schwartz *et al.*, 1990). Ubiquitin is known to play

a role in PCD of diverse tissue types (human lymphocytes, human neurons, mammalian muscle as well as *M. sexta* neurons; reviewed in Sandri *et al.*, 1997), however some tissues such as T-lymphocytes and sympathetic neurons show no ubiquitin up-regulation during PCD (Schwartz *et al.*, 1993; Martin *et al.*, 1992). The family of proteins called caspases has been shown to be the most common proteases present during apoptosis (Li *et al.*, 1997).

Muscle degeneration also occurs in the grasshopper, *Barytettix humphreysii*, following the shedding of a hindlimb by an escape response called autotomy (Arbas and Weidner, 1991). The degeneration occurs in a group of thoracic muscles that move the remaining two joints of the limb, although the muscles are neither damaged nor denervated during the escape response. Muscle degeneration is triggered transneuronally when the leg nerve, which carries both motor and sensory information, is severed during autotomy (Arbas and Weidner, 1991). Experiments from earlier work (Personius and Arbas, 1998) demonstrate that muscle breakdown after autotomy occurs by a two step process, a slower phase which may be the cessation in normal muscle growth following the molt to adulthood, and a later phase (~10 to 15 days post-autotomy) during which muscle undergo significant electrophysiological changes, rapidly lose fiber cross-sectional area and may fibers disappear altogether. Taken together, these findings suggest that PCD could be the mechanism for muscle degeneration following autotomy.

The alliterative is that muscle degeneration following autotomy occurs by muscle wasting, the mechanism thought to occur following muscle disuse, where protein synthesis is decreased and protein degradation is increased, but without the occurrence of

a specific genetically controlled program (Goldspink *et al.*, 1983). Muscle wasting may occur subsequent to autotomy, since the thoracic muscles are no longer normally loaded. After tenotomy or joint immobilization of the flexor tibiae muscles in the desert locust (*Schistocera gregria*), Jahromi and Bloom (1979) found ultrastructural changes in a subset of fibers 9 days following pertubation, while after longer periods (45-73 days) almost all of the muscle fibers showed a loss of myofilaments and replacement lipid deposits. In vertebrates, the ratio of muscle strength to cross-sectional area remains normal and no decrease in fiber number is seen during disuse (Cardenas *et al.*, 1979). Additionally, no significant changes in fiber electrophysiology occur during muscle fiber disuse in vertebrates or invertebrates (Brooks, 1970; Fischbach and Robbins, 1971; Atwood *et al.*, 1978). Since electrophysiological changes are seen in grasshopper muscle soon after autotomy, muscle wasting as a mechanism for muscle degeneration appears to be unlikely.

In this paper I address the possibility that muscle fiber degeneration subsequent to autotomy in a grasshopper involves PCD. Additionally, I hypothesize that, if muscle degeneration occurs by PCD, markers for PCD should be present only during the second phase of muscle breakdown (*i.e.* ~ 10-15 days post-autotomy). Therefore, I used histochemical and immunohistochemical techniques to study the time course of PCD death in one of the affected thoracic muscles, trochanter depressor muscle #133b,c (M#133b,c). In summary, I have found that muscle degeneration is dependent on *de novo* protein synthesis and that ubiquitin-immunoreactivity, chromatin condensation, and terminal deoxynucleotidyl transferase mediated digoxigenin-conjugated

deoxyribonucleotide triphosphate nick end labeling (TUNEL) are present in muscle fibers during the rapid phase of muscle denervation (~10-15 days). All of these finding are consistent with the occurrence of PCD.

MATERIALS AND METHODS

Experimental animals

The grasshoppers used in all experiments were from a colony of *Barytettix humphreysii*, reared on a 16:8 light:dark cycle at room air temperature. Radiant heat from 100W light bulbs during the photophase allowed animals to regulate body temperature by adjusting their position within the cage. Animals were fed romaine lettuce with wheat germ/bran mix *ad libitum*. All experimental animals (except animals used in cycloheximide experiments) were induced to autotomize a single hindlimb on the day following their molt to adult (day 0), by holding the grasshopper by a single hindlimb and gently shaking. Only one muscle (M#133b,c) of the group of degenerating muscles was examined in this study.

Cycloheximide injection

Since grasshopper muscles continue to grow in the days following their molt to adult, animals used for protein synthesis blockade experiments were induced to autotomize one month after the adult molt when muscle size is stable. Test insects received a single injection of 0.11 mg cycloheximide (Sigma) in 5 µl grasshopper

saline/ethyl alcohol, while control animals received an equal amount of solvent only. Solvents were sterilized by filtration through a 0.22 µm Nalgene syringe filter (Fahrbach *et al.*, 1993). Injections were made into the ventral abdomen on day 8 or day 13 post-autotomy, since rapid degeneration of muscle occurs during the period 10-15 days post-autotomy (Personius and Arbas, 1998), and cycloheximide appears to be active for only 48 hrs (Fahrbach *et al.*, 1993). M#133b,c was dissected out of animals on day 10 or day 15, respectively, and muscle wet weight was determined (n = 4 for saline injections; n = 6 for drug injections for each time period).

Muscle preparation

Animals were first cold anesthetized at 0°C for 10-15 mins. Dissections were performed in low Ca^{2+} saline to reduce muscle contractions. M#133b,c was removed with the cuticle at its origin and insertion intact. Muscles samples used for immunohistochemistry were prepared for cryostat cross-sections. The muscles were placed in carboxymethylcellulose and frozen in isopentane cooled by liquid nitrogen. Muscle preparations were mounted with Tissue TEK on a cold chuck (-20°C) of a cryotome (B/K instruments) and serial cross sections (20 µm) were made and placed on dry, glycerin-subbed cover slips. Whole-muscle samples for longitudinal sections were fixed *in* situ in 4% paraformaldehyde in phosphate-buffered saline (PBS) overnight, dissected out in PBS, embedded in paraffin wax (paraplast), and serially sectioned (6µm).

Ubiquitin immunohistochemistry and filamentous actin labeling

To determine whether ubiquitin was up-regulated in degenerating muscle, frozen cross-sections were double labeled for ubiquitin-immunoreactivity and filamentous actin. Cross-sections were fixed in 4% paraformaldehyde for 2 hrs and permeabilized in PBS-Tx (1% Triton-100 in PBS) several times. Nonspecific activity was blocked by immersing the sections in a solution of 30 mg powdered milk per 1 ml buffer, 0.25% bovine serum albumin and 2% normal goat serum in Tris buffer (0.05M Tris, 0.85% NaCl and 0.25% Triton-100) for 1 hr (Dr. N. T. Davis, personal communication). After washing in PBS, samples were labeled with the primary antibody (rabbit anti-ubiquitin, Chemicon) diluted 1:1000 in blocking solution overnight at 4°C. Following washing in PBS, samples were labeled with the secondary antibody (Rhodamine goat anti-rabbit, Jackson Immunoreasearch, Inc.) diluted 1:100 for 1 hr. Samples were again washed in PBS and incubated in 66nM bodipy-phallacidin (Molecular Probes, Inc.) in PBS for 2 hrs to label filamentous actin (Consoulas and Levine, 1997). Following final washings in PBS, preparations were mounted in 80% glycerol/ 0.05M carbonate-bicarbonate (n = 3 animals at each time period; 3, 10 and 15 days post-autotomy).

Labeling of nuclei

Since chromatin condensation is a common feature of PCD, longitudinal paraffin-embedded muscle sections were labeled with propidium iodide (Sigma). After deparaffinization and rehydration, sections were incubated in 25 μM propidium iodide in

PBS for 3 min (Sun *et al.*, 1993). Pretreatment with RNAase was not performed, therefore free RNA was not eliminated from the cytoplasm, and the cytoplasm was lightly stained in addition to the nuclei. Some sections were later stained with hematoxylin-eosin to better visualize the relationship between fiber cytoplasm and pyknotic nuclei. These samples, in addition to prodidium iodide labeled sections, were used to determine the % of pyknotic nuclei at 10 and 15 days post-autotomy. The number of pyknotic nuclei vs total nuclei number was determined from three sections which were at least 60 μm apart in the series and then averaged to give the % of pyknotic nuclei for that muscle. Muscle samples from the autotomized and internal control sides of 3 insects were used for both time periods.

TUNEL (TdT-mediated dUTP nick end labeling)

PCD often involves fragmentation of DNA following synthesis of the proteins necessary for destruction of the cell. TUNEL labeling was, therefore, used to determine whether DNA fragmentation occurs in muscle nuclei following autotomy. DNA strand breaks can be identified by labeling of free 3′-OH termini with modified nucleotides. TUNEL labeling uses terminal deoxynucleotidyl transferase (TdT) to add a fluorescein digoxigenin-conjugated deoxyribonucleotide triphosphate (dUTP) at DNA strand breaks. After deparaffinization and rehydration, longitudinal muscle sections from both the autotomized and internal-control sides of the insect were labeled according to the TUNEL kit instructions (Boehringer Mannheim). Basically, sections were washed in PBS,

permeabilized in proteinase K (10 μg/ml in Tris-HCl buffer), rinsed in the TdT mixture plus nucleotide mixture in reaction buffer (experimental preparations), or the nucleotide mixture in reaction buffer alone for negative control. Sections were washed in PBS and mounted in 80% glycerol/ 0.05 carbonate bicarbonate (n = 3 animals for each time period: 3, 10 and 15 days post-autotomy).

Viewing of preparations

Preparations were viewed with a confocal microscope (MRC-600 with a Nikon Optipho-2 microscope and a krypton/argon laser light source; BioRad). Two shambling channels and dichromatic cubes were used (BioRad K1 and K2: excitation wave lengths of 488 and 568nM, respectively), optical sections were recorded for one or two dyes. If two dyes were used, the images could be merged by using different pseudo-color (red for propidium iodide or Rhodamine and green for bodipy-phallacidin or FITC-dUTP). Hematoxylin-eosin preparations and nuclear counts of propidium iodide stained preparations were viewed with a Leitz microscope (Laborlux S).

Statistics

A one-tailed unpaired t-test was used to compare muscle weight wet in cycloheximide injection vs saline injection experiments on the autotomized side. Percentage data for pyknotic nuclei was first normalized by arcsine transformation and a one-tailed unpaired t-test performed. One-tailed tests were used because I hypothesized

that cycloheximide would slow muscle degeneration and the % of pyknotic nuclei would be increased following autotomy.

RESULTS

Effects of Cycloheximide on muscle degeneration

Cycloheximide injections significantly reduced the extent of muscle degeneration, when the wet muscle weight of M#133b,c on the autotomized side of animals injected with cycloheximide was compared with control injected animals (Figure 3.1). Significant effects were found at both 10 and 15 days post-autotomy following injections at 8 and 13 days, respectively. Cycloheximide did not affect the normal maintenance of muscle mass, since the internal-control M#133b,c was the same weight in cycloheximide and control injected animals.

Ubiquitin-immunoreactivity is increased following autotomy

Muscles on the un-autotomized side of the insects continued to exhibit strong filamentous actin labeling with only background ubiquitin-immunoreactivity even 15 days after autotomy (Figure 3.2A, B). The muscles on the autotomized side already showed some signs of up-regulation of ubiquitin-immunoreactivity fibers by 3 days post-autotomy (Figure 3.2C, arrow). No muscle fiber loss was seen in samples 3 days post-autotomy, and the fibers which showed increased ubiquitin-immunoreactivity were also lightly labeled for filamentous actin (Figure 3.2C, D; arrows). By 10 days post-autotomy

muscle fiber loss was evident in all preparations, with areas of heavy ubiquitin-immunoreactivity labeling showing no labeling for filamentous actin (Figure 3.2E, F; arrows). Further muscle fiber loss was seen on day 15, and large areas of muscle tissue which were unlabeled for filamentous actin were heavily labeled for ubiquitin-immunoreactivity (Figure 3.2G, H). Even within individual fibers, areas of low filamentous actin labeling show strong ubiquitin-immunoreactivity (Figure 3.2G, H; arrows).

Chromatin consolidation

Normal muscle nuclei appeared in longitudinal sections as elongated ovals when labeled with propidium iodide. Fiber striations were discernible in internal-control fibers (Figure 3.3A). By 10 and 15 days post-autotomy some fibers showed nuclei with consolidated chromatin surrounded by a hazy nuclear envelope (arrow), in addition to a few highly consolidated pyknotic nuclei (Figure 3.3B; arrowhead). Other non-striated degenerating muscle fibers showed normal, but possibly smaller, nuclei. Figure 3.4A-B shows muscle sections from an animal 10 days post-autotomy stained with hematoxylin-eosin which were used to determine the % of pyknotic nuclei. Pyknotic nuclei are clearly evident as dark round nuclei in the fibers in these sections (arrows). The onset of PCD was not uniform throughout a muscle. Figure 3.4A shows a single muscle fiber in which all nuclei were pyknotic, while other fibers contain only normal nuclei. Furthermore, the onset of PCD was not uniform even within a muscle fiber, since some fibers were found

to contain normal nuclei at one end of the fiber and only pyknotic nuclei at further points along the fiber (Figure 3.4B). Note that the fiber diameter is decreased in the area of pyknotic nuclei. The % of pyknotic nuclei was increased over internal-control samples in all animals 10 and 15 days post-autotomy (Figure 3.4C). Significant increases were found when results were grouped for each time period (day 10, 5.6±1.9 vs. 0.3±0.2; day 15, 3.6±1.0 vs. 0.3±0.2; autotomized side vs. internal-control; mean±SE).

TUNEL labeling for DNA fragmentation

In some preparations positive TUNEL labeling was found in a few muscle nuclei 3 days post autotomy (Figure 3.5B) even though little muscle degeneration was evident (note presence of fiber striations and absence of pyknotic nuclei). Muscle sections from internal-control fibers showed no positively labeled nuclei, but did show some areas of FITC auto-fluorescence (day 15; Figure 3.5A). By 15 days post-autotomy, severe degeneration was present, but most nuclei persisted. Positive TUNEL labeling was usually found in round pyknotic nuclei 15 days post-autotomy (Figure 3.5C). Ten days post-autotomy, the muscles generally showed a greater number of nuclei with positive TUNEL labeling than day 15 samples from the autotomized side (not shown).

DISCUSSION

Multiple markers for PCD (*i. e.* chromatin condensation, positive TUNEL labeling and up-regulation of ubiquitin-immunoreactivity) demonstrate that muscle

degeneration following autotomy occurs by PCD. Furthermore, since cycloheximide injections decreased the extent of muscle degeneration, autotomy induced muscle breakdown is clearly an active process. I expected some muscle degeneration to be present following cycloheximide injections, since injections were made at 8 and 13 days post-autotomy after significant muscle degeneration has already occurred (Personius and Arbas, 1998). The timing of peak ubiquitin-immunoreactivity and TUNEL labeling correlated with the timing of muscle fiber loss and rapid degeneration (~10-15 days post-autotomy; Personius and Arbas, 1998). Pyknotic nuclei and chromatin condensation were also found at this time.

As mentioned previously, during disuse atrophy the ratio of muscle strength to cross-sectional area is unchanged, loss of muscle fiber does not occur (Cardenas *et al.*, 1977), and no changes in fiber electrophysiology are seen (Atwood *et al.*, 1978). In *M. sexta*, 3 days prior to adult eclosion, the intersegmental muscles begin to degenerate, resulting in a 40% loss of muscle mass without significant changes in physiological properties. For example, the ratio of strength to cross-sectional area remains normal and striation patterns persist. After eclosion during the period of PCD, however, the ratio of strength to cross-sectional area decreases, sensitivity to intracellular calcium increases, and electrophysiological changes occur (Lockshin, 1973; Schwartz, 1992).

Muscle degeneration following autotomy in *B. humphreysii* appears to follow a similar pattern (*i. e.* muscle atrophy followed by PCD), since no fiber loss is seen 3 days following autotomy, fiber striation patterns persist and electrophysiological changes are evident in only a few fibers (Personius and Arbas, 1998). By 10 days post-autotomy,

however, electrophysiological changes are seen in almost all fibers and markers of PCD are evident (Personius and Arbas, 1998). The first phase of muscle degeneration (3 to ~10 days) may, therefore, be an atrophy response followed by the second phase (~10 to 15 days) where muscles are destroyed by PCD.

A few muscle fibers in some preparations, however, demonstrate PCD markers as early as 3 days post-autotomy when very little change has occurred in the gross appearance of M#133b,c. Even at 10 days, some fibers contained only pyknotic nuclei while other fibers within the same muscle contained only normal nuclei. Similar results were also found for ubiquitin-immunoreactivity, where 10 days post-autotomy, some fibers were heavily labeled, while other fibers showed only background ubiquitin-immunoreactivity. Therefore, it appears that the onset of PCD is quite variable following autotomy, since some animals demonstrate a loss of M#133b,c fibers and multiple markers of PCD 10 days post-autotomy, while other animals have the normal number of M#133b,c fibers (Personius and Arbas, 1998).

The variation in onset of PCD following autotomy is unlike muscle degeneration following eclosion in *M.* sexta, where all fibers within an intersegmental muscle demonstrate increased ubiquitin-immunoreactivity at the same time (Schwartz *et al.*, 1990). The signal for a muscle fiber to commit suicide following autotomy, therefore, appears to be fiber specific and unlike the *M.* sexta is not due to a general signal to the entire muscle. Uneven PCD is also seen during rat muscle development. Suprathreshold motoneuron activity has been shown to regulate the survival of developing rat muscle fibers, where the loss of excitation-contraction coupling leads to muscle fiber death

(Ashby *et al.*, 1993). It is thought that the amount of input received by a muscle fiber affects the amount of both autocrine and paracrine trophic support, and therefore, muscle survival on a fiber by fiber basis (Funakoshi *et al.*, 1995; Trachtenberg, 1998). Similar signals may also control muscle fiber degeneration subsequent to autotomy, since we have recently found the neuronal activity to M#133b,c is decreased 10 and 15 days post-autotomy. Thus, some muscle fibers may undergo PCD soon after autotomy, and possibly these fibers are receiving inadequate neuronal input to prevent them from activating an intrinsic suicide program (Tews *et al.*, 1997a,b).

Unlike the intersegmental muscles of *M. sexta*, M#133b,c showed the usual markers of PCD; positive TUNEL labeling and chromatin condensation. Developing or denervated rat muscles and limb muscles of *M. sexta* undergoing metamorphosis, also show positive TUNEL labeling and chromatin condensation (Consoulas and Levine, 1997; Tews *et al.*, 1997a,b; Trachtenberg, 1998). These nuclear changes have even recently been found in mouse models of muscular dystrophy and humans suffering from motoneuron disorder (Sandri *et al.*, 1997; Tews *et al.*, 1997a). Therefore, the thoracic muscles of the grasshopper appear to die by a process common to muscle in many species.

Figure 3.1 Cycloheximide, a translational blocker, slows the rate of degeneration following of muscle #133b,c autotomy. Dark bars represent the autotomized side muscle wet weight; open bars represent the internal-control side. Animals received injections of cycloheximide/saline/ethyl alcohol (experimental) or saline/ethyl alcohol (control) 8 or 13 days post-autotomy. Muscle wet weight was determined two days later, at 10 or 15 days following autotomy, respectively. Significant differences were seen between the autotomized side of experimental and control muscles for both 10 and 15 days post-autotomy (shown by asterisk). No differences were seen between the internal-control sides of experimental and control muscles for either time period (single tailed unpaired t-test; $n = 4$ control and $n = 6$ experimental for both time periods; $p < 0.05$).

Figure 3.1

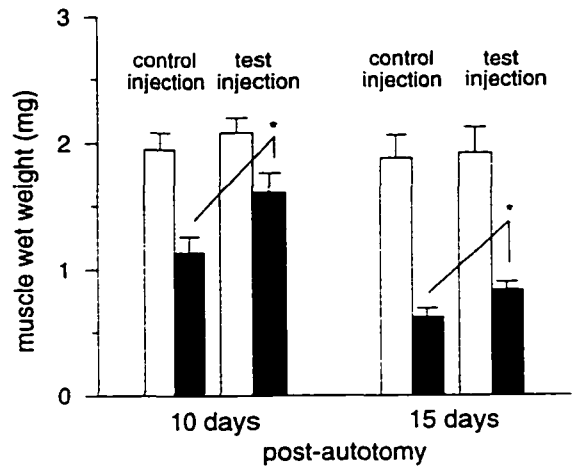

3
2
1
0
muscle wet weight (mg)
control injection
test injection
control injection
test injection
*
*
10 days
15 days
post-autotomy

Figure 3.2 Ubiquitin-immunoreactivity is up-regulated post-autotomy and areas of heavy ubiquitin-immunoreactivity show light or no filamentous actin (F-actin) labeling. Left hand panels: F-actin; right hand panels: ubiquitin-immunoreactivity from double labeled muscle #133b.c cross-sections. Panels A and B, internal-control muscle 15 days post-autotomy. Panels C and D, 3 days post-autotomy. Note that small fibers with ubiquitin-immunoreactivity also have decreased labeling for F-actin (arrows). Panels E and F, 10 days post-autotomy. Muscle fiber loss is now present and areas with no F-actin labeling show heavy ubiquitin-immunoreactivity (arrows). Panel G and H, 15 days post-autotomy. Many fibers showing ubiquitin-immunoreactivity now show no F-actin labeling. Even within individual fibers, areas without F-actin labeling show heavier ubiquitin-immunoreactivity (arrows, scale bar = 125μm).

Figure 3.2

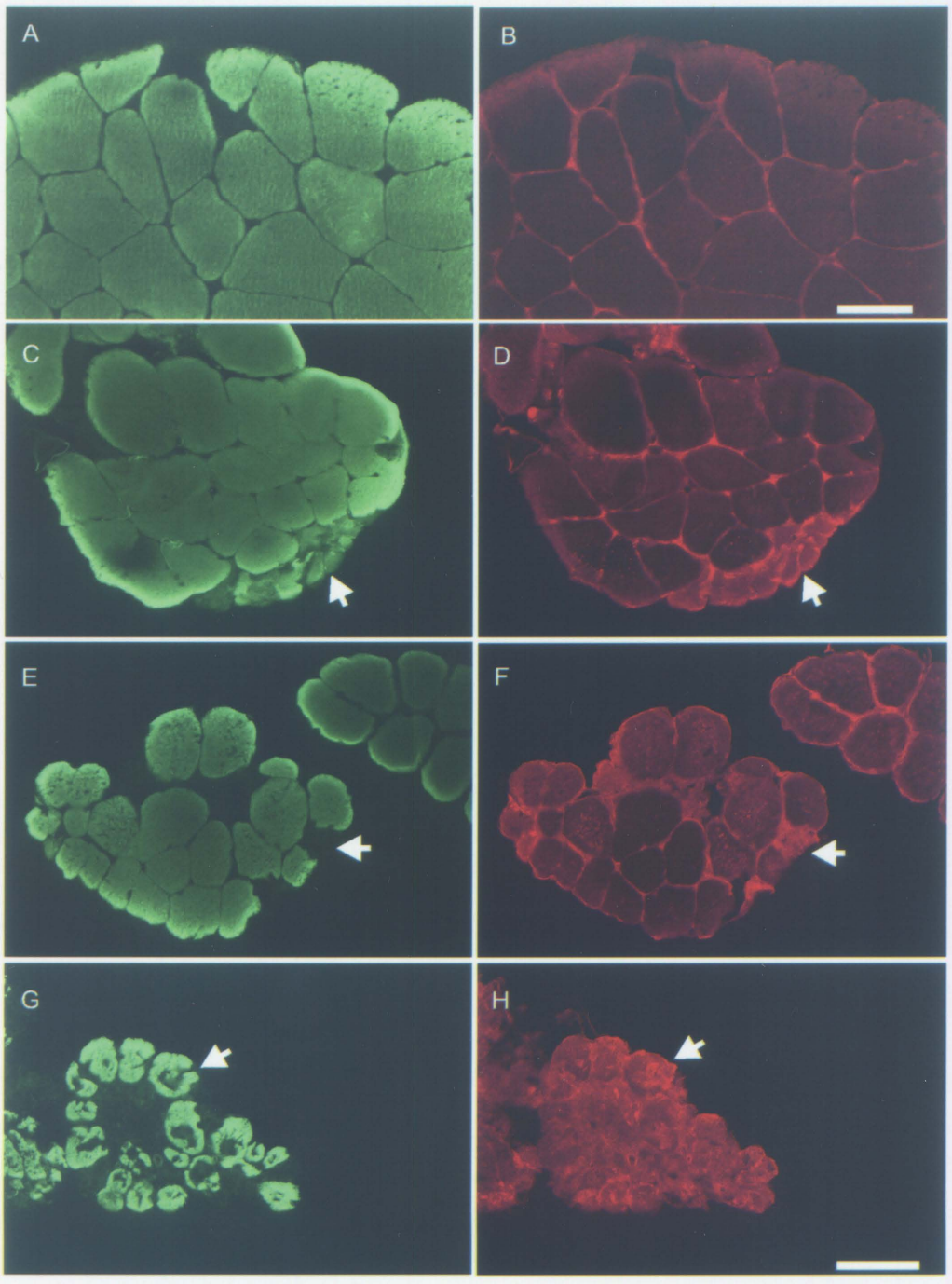


Figure 3.3 Chromatin consolidation is present in muscle #133b,c fibers on the autotomized side 10 days post-autotomy. Longitudinal-sections were labeled with propidium iodide. Muscle nuclei normally are elongated ovals in longitudinal sections and fiber striations are present in internal-control fibers (A). On the side of autotomy, muscle nuclei often show condensed chromatin within a hazy nuclear envelope (arrow) and pyknotic nuclei are present (arrowhead). Normal nuclei in non-striated fibers are also present (scale bar = 10μm).

Figure 3.3

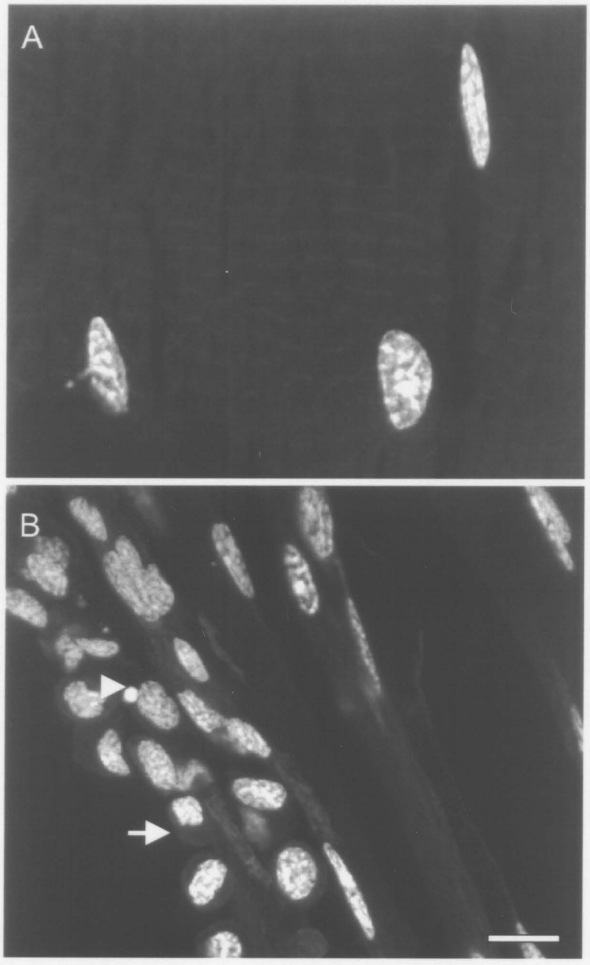


Figure 3.4 Programmed cell death is not uniform within M#133b.c or within individual muscle fibers and the % of pyknotic nuclei is increased in muscle sections from the side of autotomy compared with the internal-control side. Pyknotic nuclei are evident as dark round nuclei (arrows). Panel A shows a muscle fiber which contains only pyknotic nuclei while all other fibers, some of which are clearly striated, contain normal nuclei. Panel B shows a muscle fiber which appears normal at one end and suddenly becomes narrower with pyknotic nuclei at the other end (A, B; bar = 250μm). Panel C shows that the % of pyknotic nuclei is increased in muscle #133b.c sections from the side of autotomy (dark) compared with sections from the internal-control side (open) in all animals both 10 and 15 days post-autotomy. When results from all animals are grouped, significant increases are seen 10 and 15 days post-autotomy (single tailed unpaired t-test: $n = 3$ for both time periods; $p < 0.05$).

Figure 3.4

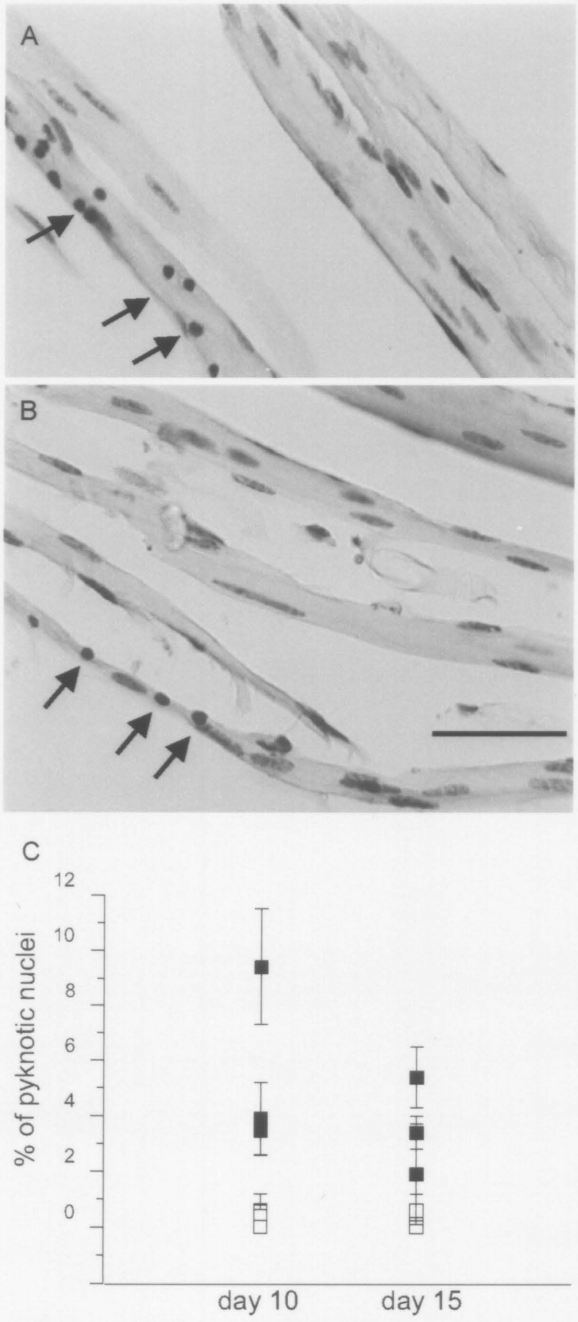
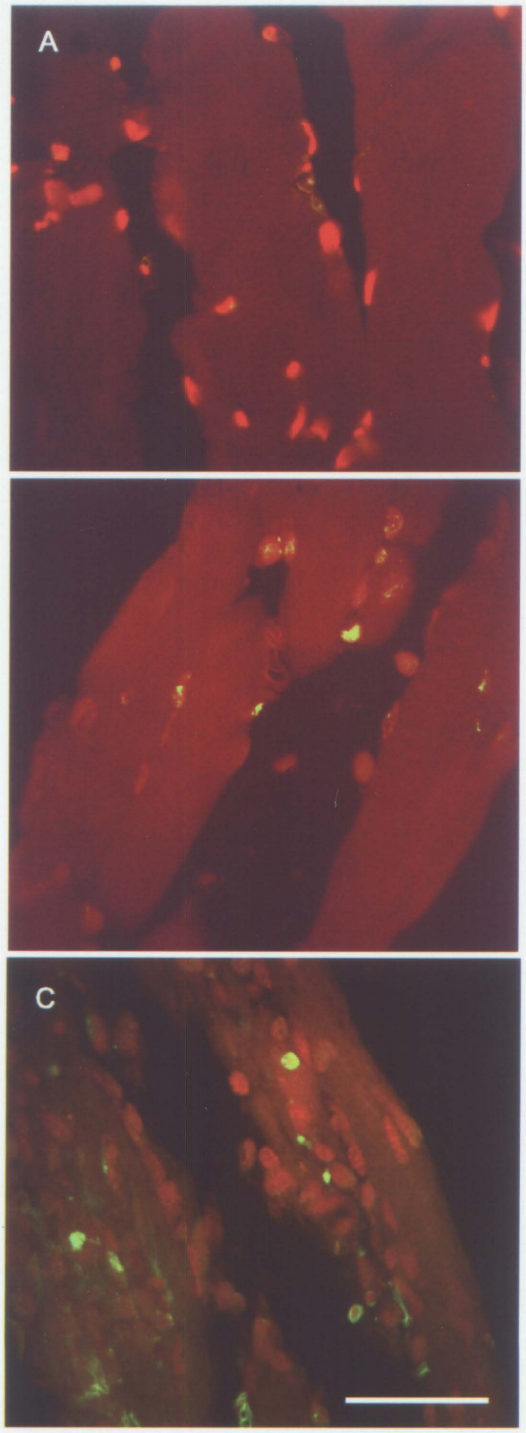


Figure 3.5 Positive TdT-mediated dUTP nick end labeling (TUNEL) is present in muscle #133b,c following autotomy. A. Muscle sections from fibers on the internal-control side did not demonstrate TUNEL labeling, however areas of FITC auto-fluorescence are present. B. By 3 days post-autotomy, some nuclei (labeled green) show positive TUNEL labeling, but pyknotic nuclei are not present. C. By 15 days post-autotomy, however, pyknotic nuclei are present and these nuclei are positive for TUNEL labeling (scale bar = 250μm).

Figure 3.5



CHAPTER FOUR: TRIGGERING MECHANISMS FOR MUSCLE DEGENERATION FOLLOWING REMOTE NERVE INJURY

ABSTRACT

Muscle degeneration can be induced by many mechanisms including denervation, loss of trophic support, loss of neuronal input, disuse, and changes in hormonal titer. In some grasshoppers, muscle degeneration is induced when the animal sheds its hindlimb during an escape response called autotomy. In this case, the thoracic muscles which degenerate are neither damaged nor directly denervated by autotomy. Muscle degeneration occurs by programmed cell death and has been shown to be transneuronally induced when the leg nerve, which does not innervate thoracic muscles, is severed during autotomy(Arbas and Weidner, 1991; Personius and Chapman, 1997).

The possible triggers of muscle degeneration following autotomy are investigated by studying the changes in one of the affected muscles, tergotrochanteral depressor muscle #133b,c (M#133b,c), and the nerve innervating M#133b,c following experimental pertubation. Degeneration of M#133b,c is correlated with a decrease in the spontaneous activity of its neuronal input. Furthermore, the population of active motor units within this nerve (N3c) is reduced. The trigger for muscle degeneration appears to be the loss of proprioceptive input, since the severing of axons from the subgenual organ leads to muscle degeneration, while the loss of exteroceptive chemo- or mechanoreceptor input, as well as the severing of motor efferents does not. Juvenile hormone and muscular

disuse also do not appear to play a role in autotomy-induced degeneration

The loss of proprioceptive input probably leads to deafferentation of the motoneurons with axons in N3c leading to a decrease in their spontaneous activity and subsequent muscle degeneration. Whether the loss of neuronal input itself to the muscle and / or the loss of activity-dependent trophic factors leads to muscle degeneration is unknown.

INTRODUCTION

Muscle is a highly plastic tissue and its mature state is modified by its genetic make-up, pattern of activity and level of trophic support (Buller and Pope, 1977; Sanes, 1987; Grinnell, 1994). Changes in muscle properties, including degeneration, can therefore be induced by denervation, changes in neuronal input, disuse, loss of trophic support and changes in hormonal titer (Redfern and Thesloff, 1971, Usherwood, 1973; Gutmann, 1976; Buller and Pope, 1977; Schwartz, 1986; Musacchia *et* al., 1988; Joubert *et* al., 1994). In the grasshopper, *Barytettix humphreysii,* muscle degeneration is induced when an animal sheds its hindlimb between the trochanter and the femur by a process called autotomy. The thoracic muscles which degenerate are neither damaged nor directly denervated during autotomy, since no muscles cross the plane of autotomy and the thoracic muscles are innervated by nerves 3 and 4 (N3 and N4; all muscle and nerve nomenclature according to Snodgrass, 1929). Arbas and Weidner (1991) have shown that muscle degeneration is transneuronally induced when the leg nerve (N5) is severed

during autotomy. The neuromuscular junction of nerve 3c (N3c) to the most severely affected thoracic muscle (M#133b,c) remains functional during the degeneration process (Personius and Arbas, 1998). Degeneration, therefore, is not a consequence of synaptic failure or muscle denervation. Additionally, muscle degeneration is probably not due to disuse, since experimental unloading of the thoracic muscle does not lead to atrophy (Arbas and Weinder, 1991).

N5 carries both motor axons from the metathoracic ganglion to the distal hindlimb and thousands of sensory axons from the chemoreceptors and exteroceptive and proprioceptive mechanoreceptors of the hindlimb (Burrows, 1992). The motoneurons within N5 have been shown to survive following the axotomy of their axons (Horridge and Burrows, 1974). In contrast, the sensory cell bodies are located in the periphery and their proximal axons degenerate soon after N5 axotomy (Horridge and Burrows, 1974; Zill *et al.*, 1980). This loss of sensory input has been shown to decrease excitatory input to metathoracic motoneurons (Horridge and Burrow, 1974). The severing of N5 sensory axons during autotomy, therefore, may deafferent the motoneurons innervating the affected thoracic muscles and lead to a relatively quiescent neuromuscular junction. The subsequent loss of normal motoneuronal input may trigger thoracic muscle degeneration. Examples of muscle degeneration induced by the loss of motoneuronal input are well documented. For example during the development of primary rat muscle, suprathreshold motoneuronal activity, or the loss of excitation-contraction coupling regulates muscle survival (Ashby *et al.*, 1993a, b). Furthermore, the amount of input to a muscle fiber appears to affect the amount of both autocrine and paracrine trophic support (Funakoshi

et al., 1995; Trachtenberg, 1998). In both vertebrate and invertebrate adult animals, denervation leads to electrophysiological changes and muscle atrophy (Gration *et al.*, 1978). Even if just activity or the release of nerve-released trophic substances is blocked, denervation-like changes occur, although the changes are not as profound (reviewed by Grinnell, 1994).

Muscle degeneration following autotomy could also be triggered by a hormonal factor. The degeneration of muscle triggered by changes in hormone titer, is part of normal development in many insects. For example, in Lepidoptera and Diptera, abdominal muscles used by the animals to escape their pupal cuticle degenerate immediately following adult eclosion (Lockshin, 1981; Schwartz and Truman, 1984; Schwartz, 1986 and Kimura and Truman, 1990). In these insects, muscle degeneration occurs by a two step process similar to that found after autotomy (Personius and Chapman, 1997; Personius and Arbas, 1998). In the hawkmoth, *Manduca sexta*, and silkmoth, *Antheraea polyphemus*, the slower first phase is triggered by a decline in the hormone 20-hydroxydecdysone (20-HE), while the second rapid phase, where muscle fibers die by PCD, is triggered by a further decline in 20-HE in *M. sexta* and a release of eclosion hormone in *A. polyphemus* (Schwartz, 1986). Abdominal muscle degeneration following eclosion in fruit fly, *Drosophila melanogaster*, follows the same time course and appears to be triggered by eclosion hormone alone. In the adult pea aphid, flight muscles degenerate by PCD following dispersal flight (Kobayashi and Ishikawa, 1994). In this case, muscle degeneration may be triggered by rise in juvenile hormone titer

subsequent to feeding upon host plants after migration (Rankin and Riddiford, 1977; Kobayashi and Ishikawa, 1993).

These examples suggest that autotomy-induced muscle degeneration could be triggered by changes in 20-HE or juvenile hormone titers. 20-HE is mainly produced by the prothoracic glands during development, but in grasshoppers these glands break-down soon after the adult molt (Chapman, 1982). In some insects abdominal oenocytes produce small quantities of 20-HE, but locust oenocytes do not produced 20-HE (reviewed by Rees, 1985). 20-HE is also produced by the ovaries of adult female grasshoppers (Hagedorn, 1983). Since muscle degeneration following autotomy occurs during development and in mature adults of both sexes, 20-HE is unlikely to be involved. Juvenile hormone is produced by the corpora allata, which do not break-down during adulthood, but become active during sexual maturity which, in these insects, occurs after becoming anatomically adult. Mordue (1977) showed that antennectomy of the desert locust, *Schistocerca gregaria*, leads to changes consistent with an increase in juvenile hormone titer so that, arguably, any injury that removes an appendage, such as autotomy, could produce a similar effect. Certain facts argue against juvenile hormone acting alone as a trigger of muscle degeneration post-autotomy. For example, sham operations with significant wounding do not lead to muscle degeneration (unpublished results). Since insects have an open circulatory system, any hormonal effect would likely be bilateral, but autotomy-induced degenerative changes are restricted to the side of autotomy (Personius and Arbas, 1998). However, a trigger in which juvenile hormone interacts with a neuronal signal cannot be ruled-out since in other cases of normally-occurring

muscle degeneration only specific muscles degenerate while the majority remain fully functional.

In this paper I investigate whether autotomy induced muscle degeneration is triggered by changes in neuronal input to muscle or decreases in juvenile hormone titer. In summary, spontaneous ensemble activity of N3c which innervates four of the affected thoracic muscles is significantly reduced during the second phase of muscle degeneration (10-15 days post-autotomy), and degeneration may be triggered by the loss of proprioceptive input from the hindlimb. Juvenile hormone does not appear to play a role in muscle degeneration following autotomy.

MATERIALS AND METHODS

Experimental animals

The adult grasshopper, *Barytettix humphreysii*, from a laboratory culture was used in most of these experiments. However, laboratory reared, adult *Melanoplus differentialis*, which demonstrates mild muscle degeneration following autotomy (Arbas, 1988), was used in the first set of experiments. Both insects are in the same subfamily, Melanopline. Animals were reared on a 16:8 light:dark cycle at room air temperature. Radiant heat from 100W tungsten light bulbs allowed the insects to regulate their body temperature by changing their position within the cage during the photophase. Animals were fed romaine lettuce with wheat germ/bran mix *ad libitum*. As necessary, animals were induced to autotomize a single hindlimb by holding the grasshopper by the hindlimb

and gently shaking, but the hindlimb of *M. differentialis* was removed by cutting, because animals would not autotomize when pinned in the dish. Only two muscles, the tergotrochanteral depressor muscle (M#133b,c) and the tergal promoter/elevator (M#118), of the group of degenerating thoracic muscles were examined in this study.

Changes in nerve 3c activity

The acute effects of autotomy on ensemble activity of N3c was studied using adult *M. differentialis*, while chronic changes were studied in *B. humphreysii*. The insects were first cold anesthetized at 0°C for 15 mins and pinned-out on a dissecting dish ventral side up. After cleaning with 95% ethyl alcohol, the ventral cuticle was removed to expose the metathoracic ganglion and N3c on each side of the animal. N3c innervates the thoracic muscles, M#121, M#130, M#133d and M#133b,c, all of which degenerate following autotomy (Figure 4.1). While superfusing with grasshopper saline, suction electrode recordings from N3c were obtained using a differential AC amplifier (A-M systems). Extracellular signals were amplified 1000-fold and filtered (60 cycle notch filter, 10Hz low cut-off, 10kHz high cut-off). Output was recorded on tape via a digital 3000A (Vetter) data recorder and later analyzed with Datapac III software (RUN Technologies). After obtaining stable a baseline recording from N3c on each side of the animal, one hindlimb was cut-off the insect along the normal plane of autotomy. Five-minute-long recordings where obtained during periods of low activity, when the insect was quiescent and not obviously struggling. Bilateral recordings were made 10 mins, 0.5, 1.0, 2.5 and 3.0 h after removal of the hindlimb (n = 3 insects). These records were later analyzed for

the total number of spikes per unit time (seconds) to determine the ensemble activity.

In the experiments with *B. humphreysii*, bilateral extracellular recording from N3c were obtained from adult animals 1, 3, 5, 10 and 15 days post-autotomy to determine whether autotomy causes long-term changes in N3 activity (n = 5 insects for each time period). In this experiment, all grasshoppers were induced to autotomize a hindlimb on the first day following their molt to adulthood (day 0). Records obtained during periods of low animal activity were used to determine whether changes occurred in the spontaneous ensemble N3c activity. To determine the maximal number of active axons in N3c, recordings were also obtained during periods of high activity as the insect struggled to move after air was blown on to its head. From these records (10-20 seconds), I was able to identify short periods (0.5-1 seconds) during which the maximal individual spike profiles were present, but no interference between different spike profiles occurred. I determined the number of different spike profiles within the trace by manually measuring differences in spike height and spike duration. This information was used to determine whether changes occurred in the population of N3c motoneuron spikes following autotomy.

Nerve cross-section

To determine the number of axon profiles within N3c and to determine whether there are structural changes in this nerve following autotomy, the metathoracic ganglion with N3c intact bilaterally was partially dissected and fixed *in situ* with 2.5% glutaraldehyde, 0.5% paraformaldehyde, 0.18 M $CaCl_2$, 0.58 mM sucrose, and 0.1 M

Phosphate buffer (Tolbert and Hildebrand, 1981). After 1 hr in fixative, the metathoracic ganglion with N3c was removed and fixed in the same solution overnight at 4° C. The ganglion was then cut in half and each half was prepared for microscopy by secondary fixation in 0.05% osmium tetroxide, followed by rinsing in buffer, dehydration through graded ethanols, and embedded in Epon/ Araldite. Parasagittal sections were cut through N3c at several positions along its length. One-micrometer-thick sections were cut on an Ultracut E (Reichert-Jung) and stained with toluidine blue (n = 3 animals)

Severing of nerve 3

To determine whether severing of nerve 3 and , therefore, directly denervating M#133b,c leads to muscle degeneration with a similar time course to that following autotomy, *B. humphreysii* were cold anesthetized at 0°C for 10-15 minutes and restrained on a dissecting dish ventral side up. After cleaning with acetone and 95% ethyl alcohol, a section of the ventral cuticle was removed and one side of metathoracic ganglion and its nerves was exposed. N3 was identified and a section of the nerve just distal to the ganglion was removed. The ventral cuticle was replaced and sealed in place with paraffin wax (paraplast). The ventral side of the insect was cleaned again with 95% ethyl alcohol and the insect warmed to room temperature before returning to its cage. Ten or 15 days following experimental axotomy, animals were again cold anesthetized and M#133b,c was removed from both sides of the insect for protein content analysis (n = 4 animals for each time period). The un-operated side M#133b,c served as an internal control and prior

to the removal of M#133b,c, the branch of N3c innervating the muscle was severed and stimulated with a Grass S11 stimulator via a suction electrode to assess for failure of the neuromuscular junction. Animals in which stimulation led to muscle contraction were discarded. Re-innervation did occur in a few insects. In two animals, sham operations were performed by opening the ventral cuticle but not severing N3. Protein content of M#133b,c was determined for all animals 10 or 15 days following the operation. To reduce mortality following axotomy that occurred with newly molted insects, all grasshoppers used in these experiments were 14 days past their last molt to adulthood prior to surgery. For comparison, autotomized animals were also 14 days past their molt to adulthood prior to autotomy.

Partial deafferentation

Partial deafferentation was achieved by removing sections of one hindlimb to determine whether deafferentation was a trigger for muscle degeneration following autotomy. Cuts were made between the tibia and tarsus, between the first and second spine of the tibia, or through the very proximal tibia (n = 2, 3 and 10 insects, respectively). The section between the tibia and the tarsus removed sensory input from the many chemo- and mechanoreceptors on the tarsus and pretarsus. No motor axons were damaged by this cut, since there are no intrinsic muscles in the tarsus. The section between the first and second spine of the tibia removed the same sensory input as the cut between the tarsus and tibia, but in addition also removed input from the exteroceptors of

the distal tibia. This mid-tibial cut also severed the motor axons of the depressor muscle of the tarsus, the levator muscle of the tarsus and one of the long depressor muscles of the pretarsus (Chapman, 1982). The section through the very proximal tibia produced the same reduction in chemoreceptors and exteroceptors sensory input as the mid-tibial cut, but proprioceptors were also affected. Immediately below the knee joint on the hindlimb of a grasshopper are two proprioceptive organs, the subgenual organ in the hemocoel and a group of campaniform sensilla in the cuticle. The proximal tibia section severed the subgenual organ in the tibia and might have also damaged the campaniform sensilla (Heitler and Burrows, 1977). The proprioceptors within the femur, however, were not damaged. Importantly, the proximal tibia section severed the same N5 motor axons as the mid-tibial cut between the first and second tibial spines. Protein content of M#133b,c and M#118 was determined 10 and/or 15 days following these three experimental ablations. Partial de-afferentiation was not well tolerated and more that 50% of the grasshoppers spontaneously autotomized the experimental hindlimb.

Changes in M#133b,c electrophysiology was also determined in insects 10 days following the proximal tibial cut (n = 3). In these animals, M#133b,c was exposed with both origin and insertion intact, and a glass support was placed under the muscle. The preparations were continuously superfused with grasshopper saline and intracellular recordings from muscle fibers were made in current-clamp mode (Axoclamp 2A, Axon Instruments) with microelectrodes (20-40 MΩ) filled with 3M K acetate. Resting membrane potential was determined in 10-15 fibers per side of an animal. In these same fibers, the presence of post-inhibitory rebound was identified by an overshooting voltage

spike after strongly hyperpolarizing a muscle fiber.

Protein content

The protein content of M#133b,c and/or M#118 was determined by the Bradford method (Bradford, 1975), which depends on spectrophotometrically quantitating the binding of Coomassie Brilliant Blue G-250 to the unknown protein and comparing this binding to that of different amounts of a standard protein (bovine gamma-globulin plus: BioRad). Standard assays contained 2.5, 5, 10, 15, 20, 30 and 40µl of bovine gamma-globulin plus, 97.5, 95, 90, 85, 80, 70 and 60µl of 0.5M NaOH, and 1000µl of Bio-Rad protein assay reagent, respectively (Bio-Rad). Standard assays were made in duplicate. Absorbance was measure at 595 nm by a BioSpec-1601 (Shimadzu) and a standard curve was determined.

After removing M#133b,c or M#118, muscle samples were dissolved in 100µl of 0.5M NaOH overnight at 56°C. Unknown assays contained 30 or 40µl of muscle sample, 70 or 60µl of 0.5M NaOH and 1000µl of Bio-Rad protein assay reagent, respectively. Absorbance was measured and protein concentration (µl protein per ml solution) was determined from the standard curve. Protein content of M#133b,c or M#118 was then calculated. Spectrophotometric measurements were made 10-30 minutes after addition of Bio-Rad reagent.

Blockade of juvenile hormone production

Precocene II (Sigma-Aldrich) is a plant-derived compound which has been shown

to cause severe atrophy of the corpora allata and inhibit juvenile hormone production in the locust, *Locusta migratoria* (Pener *et al.*, 1978). I used this compound to determine whether juvenile hormone is a trigger for autotomy-induced muscle degeneration. Test insects received a single injection of 100µg of precocene II in 2µl of acetone, while controls received an equal amount of acetone only. Injections were made into the abdomen on the first day following the animal's molt to adulthood. Two weeks following the injections, animals were induced to autotomize a single hindlimb. Protein content of M#133b,c was determined bilaterally 15 days following autotomy (n = 4 control animals and 7 experimental animals). To ensure that precocene II was active in destroying the corpora allata of *B. humphreysii*, an insect was sacrificed two weeks following injection. The corpora allata were found to be missing as shown by Pener *et al.* (Pener's Figure 2, 1978).

Statistics

A one-way analysis of variance (ANOVA) with Bonferroni correction was used to compare the chronic changes in the ratio of N3c ensemble activity (experimental vs. control) at various times following autotomy and to compare changes in protein content following autotomy, denervation, and partial deafferentation 15 days post-autotomy. A two-tailed unpaired t-test was used to compare the acute changes in ratio of N3c ensemble activity during the first and last time points (0 vs. 3.0 hrs), the number of different spike sizes following autotomy (autotomized vs. control), and protein content of M#133b,c at 10 days following autotomy and axototomy

RESULTS

Changes in nerve 3c activity

Immediately following the severance of a hindlimb (and N5) in *M. differentialis* there was no change in the resting ensemble activity of N3c, but within a hour the activity of N3c, on the same side as the severed leg, began to increase compared to the contralateral N3c recordings (Figure 4.2). Three hours after severing of the hindlimb, the ratio of activity (the number of spikes recorded per unit time for N3c on the same side as the severed hindlimb vs. the contralateral N3c firing frequency) was significantly greater than the ratio of activity prior to severing the hindlimb (Figure 4.2; note log scale). The population of active motor axons did not change during the 3 hour recording on either side of the animal (data not shown).

The resting ensemble activity of N3c on the side of autotomy was unchanged in *B. humphreysii* 1, 3 and 5 days following autotomy, when compared to the firing frequency of the contralateral N3c (Figure 4.3; note log scale). By 10 days and 15 days post-autotomy, however, the ratio of ensemble N3c activity between the two sides of the animal was significantly reduced from the ratio obtained 1 day following autotomy. As the insects aged following their molt to adulthood, the population of spike profiles during periods of high activity increased, possibly because N3c itself larger. (Figure 4.4A). The population of spike profiles on the side of autotomy, conversely, decreased as the insect aged. Significant differences were seen between the two sides of the insects at 10 and 15 days post-autotomy. By 15 days the population of spike profiles was significantly

reduced on the autotomized side compared to the autotomized side 1 day following autotomy (Figure 4.4B).

Cross section of N3c

N3c contains 10 motor axons and a very small few sensory axons. This number of motor axons matches well with our recordings which usually demonstrated seven different spike profiles on the control side 15 day after autotomy (Figure 4.5A). N3c does not show degenerative changes 15 days after autotomy, but changes are seen at 25 days (Figure 4.5B). These changes include altered axons (note irregular axon outlines and apparent fusion of adjacent axons) and dense staining of the neural lamella.

Protein content of muscle following autotomy, denervation or partial-de-afferentiation

As previously shown by wet-weight and fiber cross-sectional area measurements, M#133b,c degenerated following autotomy (Personius and Arbas, 1998). By 10 days post-autotomy, the protein content of M#133b,c on the autotomized side was reduced to 0.772±0.056 of control and further reduced to 0.339±0.075 by 15 days post-autotomy (mean±SE; Figure 4.6). Degeneration of M#133b,c also occurred following severing of N3 which denervated M#133b,c. The final degree of degeneration was similar to that found following autotomy, with of protein content of the autotomized muscle reduced to 0.355±0.172 of control after 15 days. The rate of degeneration, however, was faster following denervation of M#133b,c than following autotomy, since at 10 days post-

denervation the protein content was less than after autotomy (0.416±0.126 versus 0.772±0.056; Figure 4.6). Sham operated animals demonstrated no degeneration of M#133b,c 15 days after surgery (1.05±0.188 of control; Figure 4.6).

Insects deafferentated by severing the hindlimb between the tibia and tarsus or between the first and second tibial spines (long tibia) demonstrated no degeneration of M#133b,c (protein content was 0.925±0.195 and 1.04±0.066 of control, respectively). Animals that were partially deafferentated by proximal tibial sections (short tibia), however, did demonstrate muscle degeneration. The degree of degeneration was similar at 10 and 15 days following the proximal tibial section (0.703±0.117 and 0.709±0.092 of control, respectively; Figure 4.6), while after autotomy, muscle content was reduced to 0.772±0.056 of control at 10 days and 0.339±0.075 by 15 days. When the ratio of protein content was compared, 15 days after autotomy with 15 days following severing of the proximal tibia, a significant difference was seen. A significant difference in protein concentration was also seen between insects with a proximal tibial section and those severed between the first and second tibial spine (short vs. long tibia; Figure 4.6).

Electrophysiological changes similar to those found following autotomy were also found following the proximal tibial sections (Personius and Arbas, 1998). For example, the average muscle fiber resting membrane potential was significantly reduced 10 days following tibial sections when compared to internal-control fibers (50.7±2.5 vs. 60.8±1.4 mV; autotomized side vs. internal control; mean±SE; $p < 0.05$; one tailed paired t-test; n = 3 animals). Additionally, post-inhibitory rebound was found in ~ 50% of the muscle

fibers on the severed side.

M#118 also showed reductions in the amount of protein concentration following autotomy and proximal tibial sections, though the changes where not as extreme. By 10 days post-autotomy, protein content was relatively unchanged at 0.891±0.23 of control, but was reduced to 0.554±0.113 of control at 15 days. Following proximal tibial section, the reduction in M#118 protein concentration was similar to that found in M#133b,c. Thus, reductions were the same at 10 days and 15 days (0.804±0.062 and 0.758±0.045, respectively).

Prococene II injections

Insects injected with prococene II plus solvent or solvent alone both demonstrated degeneration of M#133b,c 15 day post-autotomy. The degree of degeneration in both cases was similar to that found following autotomy-alone (0.353±0.031 and 0.248±0.054 vs. 0.339±0.075; injections of prococene II and solvent alone vs. autotomy; Figure 4.7) although the corpora allata were completely absent in animals injected with prococene II.

DISCUSSION

These experiments have shown that severing N5 either experimentally or as a consequence of autotomy leads to both acute and chronic changes in the activity of N3c, a nerve which innervates four of the affected thoracic muscles including M#133b,c. The relative increase in activity of N3c acutely following the severing of N5 may be due to the

loss of inhibitory input from the injured sensory afferents (Burrows, 1992; Figures 4.1 and 4.2). Conversely, the relative activity of N3c is significantly reduced 10 and 15 days following autotomy (Figure 4.3). Since the muscle fibers of M#133b,c have been shown to undergo PCD during this period, the reduction in activity or activity-dependent trophic factors may allow muscle fibers to activate an intrinsic suicide program (Tews *et al.*, 1997a,b). The loss of adequate neuronal input has been shown to reduce the survival of developing rat muscle fibers (Ashby *et al*, 1993a). In vertebrate neuromuscular systems both muscle contraction itself and activity-dependent trophic factor release have been shown to regulate muscle degeneration. For example, blocking excitation-contraction coupling prevents the maintenance of primary muscle fibers (Ashby *et al*, 1993b), and during rat muscle development neuregulin has been shown to increase muscle fiber survival, while the amount of neuregulin at the neuromuscular junction may be controlled in an activity-dependent manner (Trachtenberg, 1998).

Whether neuronally induced muscle contraction or activity-dependent trophic factors are ultimately responsible for the maintenance of muscle in the grasshopper is unclear. This question might be answered if one was able to selectively block either the propagation of action potentials or trophic factors along N3, or directly stimulate an affected thoracic muscle following autotomy as has been performed in vertebrates (reviewed by Grinnell, 1994). These experiments, however, would be technically very difficult. Evidence for trophic factors acting at the neuromuscular junction in invertebrates is increasing. Cultured embryonic cells from *Drosophila* have been shown to produce a factor similar to nerve growth factor (NGF; Hayashi *et al.*, 1992) and

receptors similar in sequence to neurotrophin receptor kinases have been identified in the developing nervous system (Gorczyca *et al.*, 1993).

The trigger for the reduction in motor axon activity and subsequent muscle degeneration appears to be the deafferentation of the motoneurons innervating the affected thoracic muscles. Partial deafferentation by a section through the proximal tibia (short tibia) led to muscle degeneration, while sections between the tibia and tarsus, and between the first and second tibial spines (long tibia) did not lead to muscle degeneration (Figure 4.6). The loss of sensory input from the chemo- and mechanoreceptors of the tarsus and the tibia, therefore, does not affect the maintenance of thoracic muscle, nor does damage to the motoneurons innervating the muscles in the tibia since the short and long tibial cuts severed the same motor axons. However, the section through the proximal tibia, which severs the nerve for the subgenual organ and may damage the tibial campaniform sensilla, does induce degeneration. These proprioceptors, unlike the exteroceptors, are more likely to have mono-synaptic connections with the motoneurons to the affected thoracic muscles (reviewed by Burrows, 1992). Since only sections through the proximal tibia lead to muscle degeneration, it appears that the loss of proprioceptive input is particularly important in triggering muscle degeneration. Villalobos and Arbas (1988) also suggested that muscle degeneration is triggered by motoneuronal deafferentation, when they found that the motoneurons innervating M#133b,c exhibited reduced dendritic arbors 40 days following autotomy.

M#133b,c, a depressor of the hindlimb, was unloaded after sections through the mid and proximal tibia (the muscle was unloaded because the insects no longer took

weight on the hindlimb following these procedures). Muscle degeneration, therefore, could be a result of disuse. Three experimental results argue against this possibility. First, M#118, a tergal elevator and promoter, was not unloaded, but was found to degenerate following severing of the proximal tibia. Second, disuse atrophy is not associated with electrophysiological changes (Atwood *et al.*, 1978), but M#133b,c demonstrated electrophysiological changes after the proximal tibia was severed. Third, even though both sections through the tibia would unload M#133b,c only the proximal section induced muscle degeneration.

Denervation of M#133b,c ultimately led to the same degree of muscle degeneration as autotomy, but the rate of muscle degeneration was faster following axotomy (Figure 4.6). This suggests that axotomy, which removes all neuronal input and any putative trophic support, is a more significant insult to the muscle than autotomy. Thus, axotomy may more quickly activate PCD. Partial deafferentation does not lead to the same degree of muscle degeneration at 15 days as autotomy (Figure 4.6). At 10 days, however, the amount of degeneration is similar to that after autotomy, suggesting that partial de-afferentiation does not fully activate the degenerative process.

Muscle degeneration following autotomy does not appear to be triggered by increases in hormone titer, since 20-HE is very unlikely to trigger muscle degeneration and precocene II injections have no effect on the degree of muscle degeneration following autotomy. In summary, these experimental results support the hypothesis that muscle degeneration is triggered when the afferent input from specific proprioceptors is lost. The degeneration of these sensory axons leads to the de-afferentiation of the ipsilateral

motoneurons within the metathoracic ganglion and a subsequent decrease in motoneuronal activity. Finally, as occurs during vertebrate development and disease, the loss of neuronal input results in muscle fibers degenerating by PCD. It is important to note that much of this evidence is correlative and not causative. Further experiments in which specific proprioceptive structures are ablated and the effects ablation upon motoneuron activity and structure are indicated.

Figure 4.1 A schematic drawing of the metathorcic ganglion and possible neuronal interactions affected by autotomy. Autotomy severs the leg nerve (N5) which carries both motor axons to the distal hindlimb muscles and sensory axons from the leg chemo- and mechanoreceptors. Most leg afferents terminate on metathoracic interneurons (open circle), but a few terminate directly on motoneurons (filled circles). The thoracic muscles which degenerate are innervated by nerves 3 and 4. Nerve 3c (N3c) which innervates four muscles is shown.

Figure 4.1

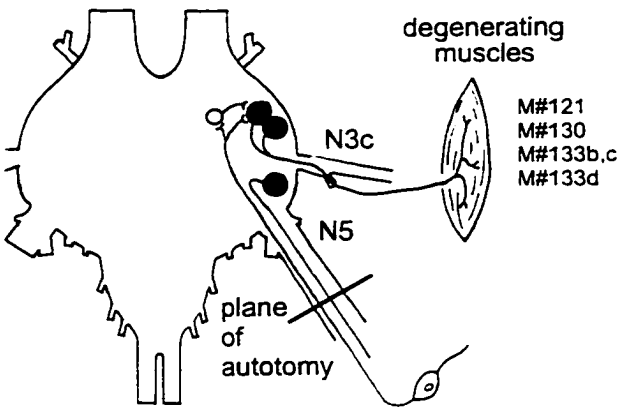

degenerating
muscles
M#121
M#130
M#133b,c
M#133d
N3c
N5
plane
of
autotomy

Figure 4.2 The ensemble activity of nerve 3c is increased soon after the severing of the leg nerve (N5) in the grasshopper, *M. differentialis*. Activity in nerve 3c on the side of axotomy is expressed relative to the activity of the contralateral side (log scale). Records were obtained during periods of low insect activity. A significant increase in relative activity was seen after three hours ($n = 3$; *, $p < 0.05$).

Figure 4.2

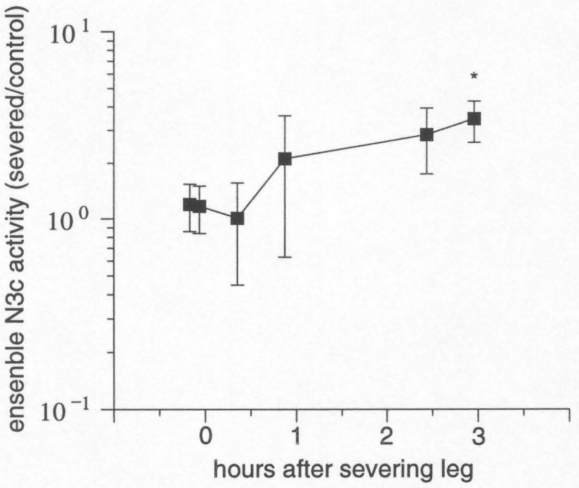

ensenble N3c activity (severed/control)
10^1
10^0
10^{-1}
*
0
1
2
3
hours after severing leg

Figure 4.3 The ensemble activity of nerve 3c in the days following autotomy. Activity in nerve 3c on the side of autotomy is expressed relative to the activity of the contralateral side (log scale). Records were obtained during periods of low insect activity. A significant decrease in relative activity occurred 10 and 15 days following autotomy in the grasshopper, *B. humphreysii* ($n = 6$; *, $p < 0.05$).

Figure 4.3

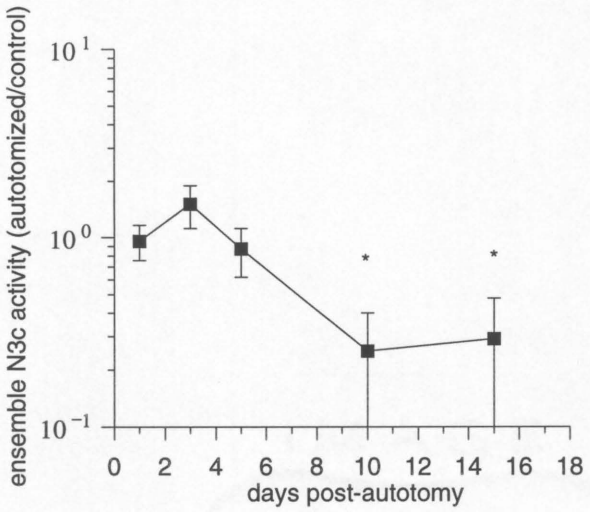

ensemble N3c activity (autotomized/control)
10^1
10^0
10^{-1}
0 2 4 6 8 10 12 14 16 18
days post-autotomy
*
*

Figure 4.4 Autotomy resulted in a reduced population of spike profiles following autotomy in the grasshopper, *B. humphreysii*. A. The number of different spike profiles increased on the internal-control side as insects aged following their molt to adulthood (open squares). while the population of spike profiles was reduced on the autotomized side (dark squares). The differences between the two sides were significant 10 and 15 days post-autotomy ($n = 6$; *, $p < 0.05$). B. Demonstrates sample records from the internal-control side (above) and autotomized (below; numbers indicate different spike profiles). Records were obtained during periods of high insect activity.

Figure 4.4

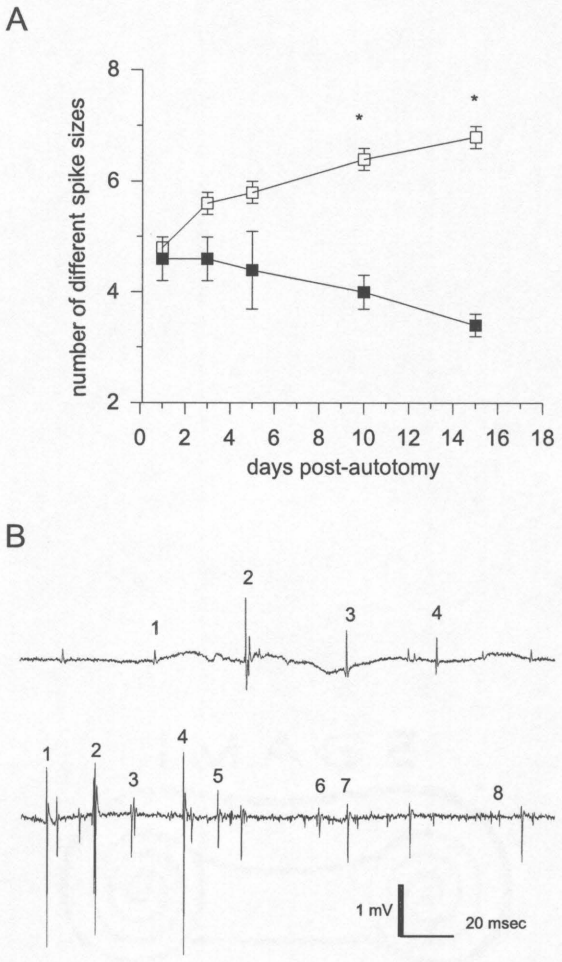

A
number of different spike sizes
8
6
4
2
0 2 4 6 8 10 12 14 16 18
days post-autotomy
*
*
B
1
2
3
4
1
2
3
4
5
6 7
8
1 mV
20 msec

Figure 4.5 A. Nerve 3c contains 10 motor axons and a few very small sensory axons. B. Degenerative changes are evident 25 days following autotomy, including irregular axon outlines, apparent fusion of adjacent axons, dense staining of the neural lamella, and possible degeneration of the axon itself. Both nerve sections are from the opposite side of the same animal and were fixed at the same time (Scale bar = 20 μm).

Figure 4.5

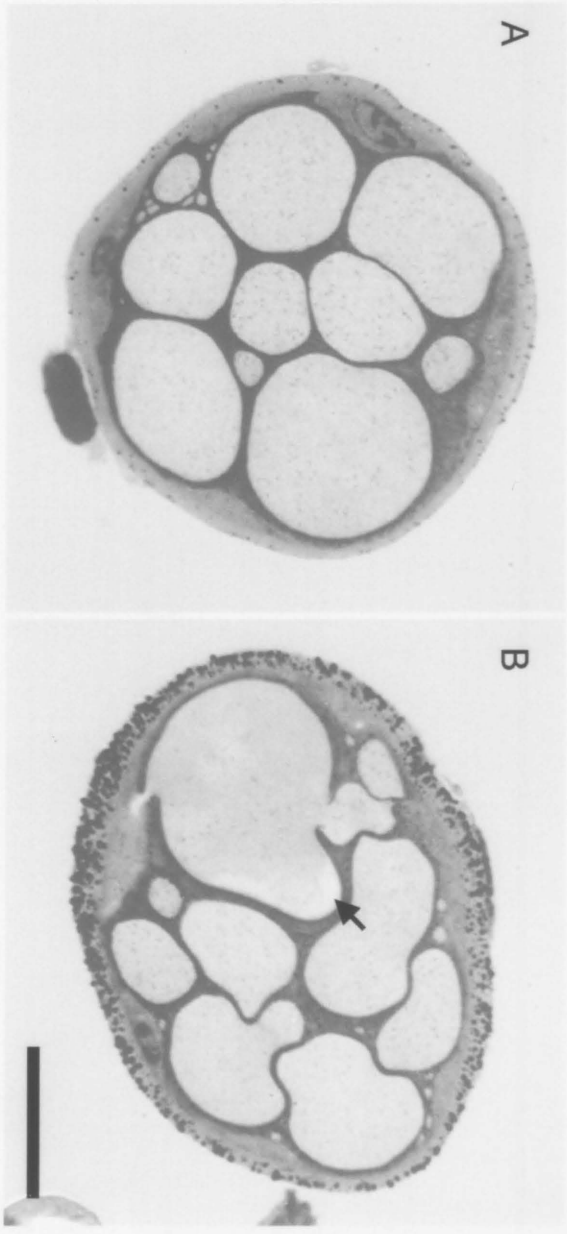


Figure 4.6 Protein content of M#133b,c after various treatments (autotomy, sham operation, denervation of M#133b,c, and partial deafferentation). The protein content of muscle #133b,c on the experimental side is expressed relative to the contralateral-control side. Sham operations (M#133b,c was not denervated) and sections through the tibia between the first and second spines (long tibia) produced no muscle degeneration. 10 days following treatment, a significant between autotomized and denervated insects was seen ($n = 4\text{-}6$; *, $p < 0.05$). Significant differences were also seen at 15 days between autotomized and short tibia cut animals and between animals with short and long tibial cuts ($n = 3\text{-}4$; *, $p < 0.05$).

Figure 4.6

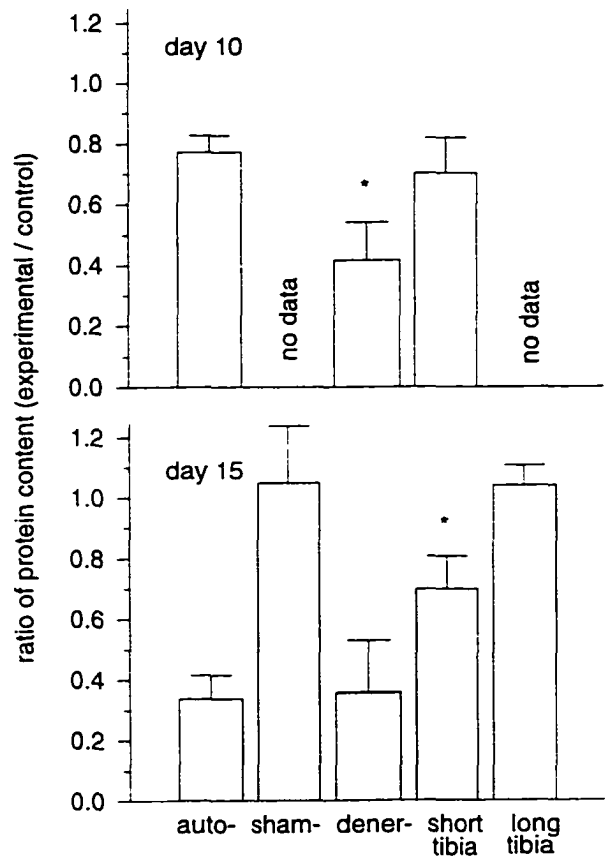
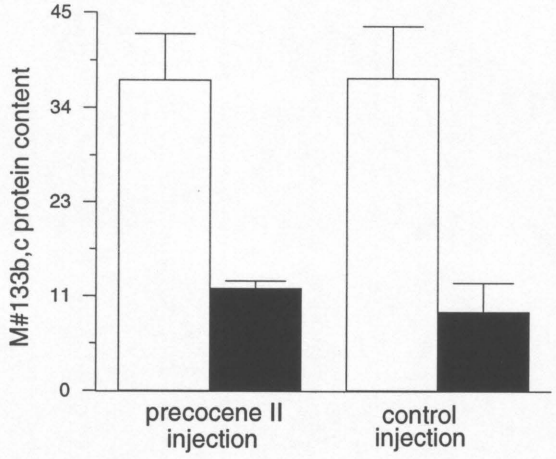

day 10
day 15
no data
no data
*
*
ratio of protein content (experimental / control)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
auto-
sham-
dener-
short tibia
long tibia

Figure 4.7 Precocene II injections did not affect the degree of muscle degeneration following autotomy. Experimental animals were injected with precocene II and solvent, while control animals were injected with solvent only. Insects received injections 14 days prior to autotomy. Protein concentration of muscle #133b,c (M#133b,c) was assessed 15 days post-autotomy.

Figure 4.7


45
34
23
11
0
M#133b,c protein content
precocene II injection
control injection

Chapter five: General discussion

The results presented in this dissertation have demonstrated that autotomy-induced degeneration of M#133b,c in newly molted adult grasshoppers occurs by a two step process (Chapter 2). The first phase, during which muscle fibers fail to grow, occurs between 3 and 10 days following autotomy (Chapter 2). The mechanism for this failure in normal growth is unclear, but presumably involves a decrease in the ratio of protein synthesis to protein degeneration (Goldspink *et al.*, 1983). During the second phase, occurring between 10 and 15 days post-autotomy, the cross-sectional area of individual fibers as well as the number of fibers within M#133b,c is significantly reduced (Chapter 2). This second phase is also characterized by 'denervation-like' changes in fiber electrophysiology including a depolarized fiber resting membrane potential, the occurrence of post-inhibitory rebound, smaller evoked excitatory junctional potentials, and an increased frequency of spontaneous miniature potentials (Chapter 2). The rapid muscle breakdown, found during the second phase of degeneration, occurs via PCD. Indicators of PCD, which include increased ubiquitin-immunoreactivity, chromatin consolidation, and DNA fragmentation, are all present 10 and 15 days following autotomy (Chapter 3) The onset of PCD is variable between individual muscle fibers, with a few fibers displaying ubiquitin-immunoreactivity at 3 days, while other fibers are relatively intact 10 days post-autotomy. This finding indicates that PCD is initiated on a fiber by fiber basis and not by a general signal to the entire muscle.

In chapter one, I discussed the six possible triggers of autotomy-induced muscle degeneration. The first, that muscle degeneration may be caused by disuse following the loss of the hindlimb, was addressed by Arbas and Weidner (1991). These researchers severed the tendon of the tibial extensor muscle, making the leg inoperable for walking and jumping, and found no muscle degeneration following tenotomy. This result indicates that disuse does not play a role in autotomy-induced degeneration. The experiments presented in this dissertation have also concluded that degeneration following autotomy is not secondary to disuse. The electrophysiological changes in muscle fibers found after autotomy are not consistent with disuse atrophy (Brooks, 1970; Fischbach and Robbin, 1971). Furthermore, the unloading of M#133b,c after removing the distal $2/3^{rds}$ of the tibia and tarsus did not lead to muscle degeneration (Chapter 2 and 4).

The second possibility, that muscle degeneration is triggered by denervation, was discounted when fibers with significant electrophysiological changes were found to have intact neuromuscular junctions (Chapter 2). The third possibility, that multiaxonal neurons carry a damage signal directly to the muscle, was negated by the findings of Villalobos and Arbas (1988), when they demonstrated that M#133b,c was not innervated by multiaxonal neurons with axons within the leg nerve (N5). The fourth possibility suggested that axotomized motoneurons within N5 may undergo changes that could have retrograde influences upon the central neurons within the metathoracic ganglion, and eventually effect the motoneurons innervating the thoracic muscles. This possibility was ruled-out by a series of experiments in chapter 4, when I severed the tibia between the

first and second tibial spine, or I severed the tibia just below the knee joint. Both procedures severed the motor axons of the depressor muscle of the tarsus, the levator muscle of the tarsus and one of the long depressor muscles of the pretarsus, but only the proximal tibial section led to muscle degeneration. Thus, the severing of motor axons within N5 does not appear to trigger thoracic muscle degeneration.

The last possibility, that a humoral factor acts as an intermediary to muscle degeneration, could only be partially ruled-out. In chapter 4, precocene II, which destroys the corpora allata, was injected into insects to determine whether increases in juvenile hormone titer were necessary for autotomy-induced degeneration to occur. Precocene II injected animals demonstrated the same degree of muscle degeneration as control-injected animals, showing that juvenile hormone does not play a role in autotomy-induced muscle degeneration. As discussed in chapter one, ecdysteroids can be partially ruled-out, since autotomy-induced degeneration occurs in mature adult animals of both sexes after the principle gland which produces ecdysteroid, the prothoracic gland, has degenerated. In some insects, very small quantities of ecdysteroids can also be produced by abdominal oenocytes, but the abdominal oenocytes of the locust, *Locusta migratoria*, cannot produce ecdysteroids (Reviewed by Rees, 1985). The ecdysteriods, therefore, can not be fully ruled-out, but the chance of them playing a role in autotomy-induced muscle degeneration is very small.

The fifth possibility, that de-afferentiation triggers autotomy induced muscle degeneration, appears to by true. Experiments in chapter 4 demonstrated that loss of proprioceptive input to the metathoracic ganglion triggers muscle degeneration, since the

severing of the subgenual organ afferents and possibly the campaniform sensilla afferents of the knee joint leads to muscle degeneration. The loss of exteroceptive chemo or mechanoreceptor input, however, does not lead to degeneration. This loss of proprioceptive input presumably leads to the de-afferentiation of the motoneurons innervating the affected thoracic muscles, since the sensory axons within the severed N5 are known to degenerate and excitatory inputs to the metathoracic motoneurons are known to decrease (Horridge and Burrows, 1974). Furthermore, following autotomy the activity of N3c is reduced. As discussed in chapter 4, N3c is one of the nerves innervating the affected thoracic muscles. Presumably as a consequence of this diminished activity, all of the muscles innervated by N3c degenerate (M#121, M#130, M#133b,c and M#133d; Chapter 4)

The extent of degeneration in the muscles innervated by N3c is variable. For example 30 days following autotomy, the protein content of M#133b,c is reduced to 4.6 % of the contralateral side, while M#130 is only reduced to 25.2 % of the contralateral side (Clinton, 1998). Variability in muscle degeneration, both between muscles and between fibers within individual muscles, is seen in all 18 of the thoracic muscles that move the remaining two segments of the hindlimb following autotomy (Clinton, 1998). If a single event, proprioceptive de-afferentiation, is the trigger for the degeneration of all of the thoracic muscles, why is there such a large variability in response to autotomy?

The dissertation work of Clinton (1998) helps to answer this question. She reports that the variability in muscle response to autotomy may be explained by muscle fiber type and innervation pattern. An example of muscle fiber type affecting the degree

of muscle degeneration, is the atrophy of M#121 following autotomy. M#121, which is innervated by N3c, degenerates to 16.4 % of its contralateral side in 30 days. Some fibers within this muscle, however, appear unaffected by autotomy. When muscle fibers are stained for myosin ATPase, all of the 'slow-type' muscle fibers are found to remain, while only a few small 'fast-type' fibers persist. A similar variability based on fiber type is seen in M#120, where 'slow-type' muscle fibers remain and 'fast-type' fibers are destroyed (Clinton , 1998; see her Figure 3.3). Since M#133b,c is composed only of 'fast-type' fibers, the work of Clinton would suggest that all M#133b,c fibers would degenerate, and experimental work in chapter two found that all M#133b,c fibers show degenerative changes. M#118, which contains only 'fast-type' fibers, responses in a similar manner with all fibers showing degenerative changes following autotomy (unpublished results).

The degree of muscle degeneration following autotomy is also correlated with innervation pattern. Immunohistochemical studies by Clinton (1998) demonstrate a correlation in the extent of muscle degeneration with innervation by a pool of motoneurons, most likely slow excitatory neurons, that contain a proctolin-like substance. The pentapeptide proctolin is one of the variety of neuromodulatory substances found in insects, and has been shown to affect muscle in a manner similar to CGRP in mammals (reviewed by Orchard *et al.*, 1989). In locusts, proctolin has been shown to increase basal tone, increase both the force and frequency of muscle contraction, and increase the amplitude of neuronally-evoked contractions (Lange and Orchard, 1984; Belanger and Orchard, 1993). Thus, when proctolin is co-released with glutamate at the neuromuscular

junction, muscle tension is increased over that induced by glutamate alone (Orchard *et al.*, 1989). Unlike CGRP, proctolin has not been shown to have trophic effects on neurons or muscle. The muscles which show the greatest degree of degeneration following autotomy (Clinton, 1998; see her Table 3.2) do not contain proctolin-immunoreactivity. As the frequency of proctolin-immunoreactivity increases so does the extent of muscle survival (Clinton, 1998), suggesting that proctolin-containing nerves may have trophic effects upon the muscle fibers they innervate.

Inhibitory motoneurons which innervate multiple muscle fibers and multiple muscles are present in many invertebrates (first documented by Wiersma, 1941). Hale and Burrows (1985) found that 13 metathoracic leg muscles in the locust were innervated by one of the three common inhibitory neurons. They also demonstrated that these inhibitory neurons innervate muscle fibers in parallel with slow excitatory motoneurons. The muscles and fiber -types innervated by common inhibitory neurons suggest that these nerves may control posture and the transition to locomotion (Schmidt and Rathmayer, 1993; Clinton, 1998). Clinton (1998) found a similar correlation between muscle innervation by the common inhibitory motoneuron #1 and muscle survival as between muscle innervation by proctolin-containing motoneurons and muscle survival. Using immunohistochemical techniques, she found that the muscles which show the greatest degree of degeneration following autotomy are not innervated by a common inhibitory motoneuron, while the degree of muscle survival increases as the frequency of common inhibitory innervation increases (Clinton, 1998; see her Table 2.3). For example, M#133b,c which shows the greatest extent of degeneration is not innervated by common

inhibitory neuron #1. Thus, it appears that the slow thoracic muscle fibers, which tend to be innervated by proctolin containing slow motoneurons and common inhibitory neurons, are resistant to autotomy-induced degeneration. This resistance may come from a variety of sources. For example, slow muscle fibers themselves may be resistant, proctolin supplied by slow motoneurons may provide trophic support, or common inhibitory input may positively affect muscle survival.

Another possible cause of variation in response to autotomy, is that the loss of proprioceptive input may have variable effects on different thoracic muscles. Bräung and Hustert (1985a,b) examined the acute effect of proprioceptor organ ablation (~1 hr) on the spontaneous activity of motor units of metathoracic muscles and their reaction to movement of the coxo-trocanteral joint in the locust, *Locusta migratoria*. They found that the loss of proprioceptive input caused no acute change in the spontaneous activity of muscles M#133b,c, M#129, M#123, M#119 and M#131, the five most degenerated muscles following autotomy. Other thoracic muscles which are less effected by autotomy, however, showed acute increases in spontaneous activity. Exceptions do occur, since motor units of both M#128 and M#118, which show only moderate degeneration following autotomy, also show no change in spontaneous activity after proprioceptor organ ablation. In chapter 4, I found the ensemble activity of N3c, which innervates M#121, M#130, M#133b,c and M#133d to be unchanged 1 hour after severing N5, activity was increased by 3 hours. Bräunig and Hustert (1985a,b) also found the activity of the axons to these muscle to be unchanged following the ablation of all proprioceptive input from N5, as well as the subcoxal receptors of N2 and N3b. The

work of Bräunig and Hustert (1985a,b), therefore, suggests an activity-dependent model to explain the differential response to autotomy displayed by most thoracic muscles, which is well correlated with the dissertation work of Clinton and myself. Thus, it appears that different levels of plasticity exist within the hindlimb motor system. In the end, the survival of an individual muscle fiber following autotomy is dependent upon fiber type, innervation pattern and the connectivity between motor units and proprioceptive input.

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IMAGE EVALUATION
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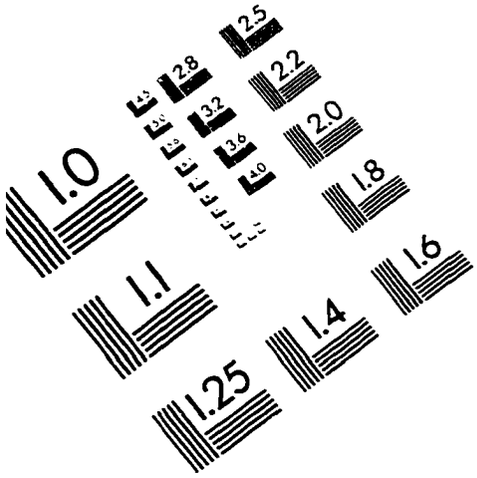

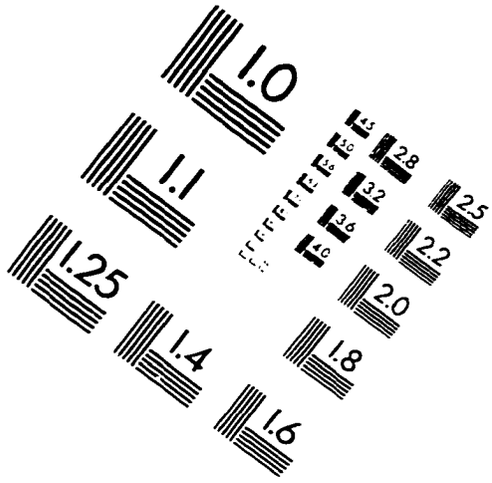

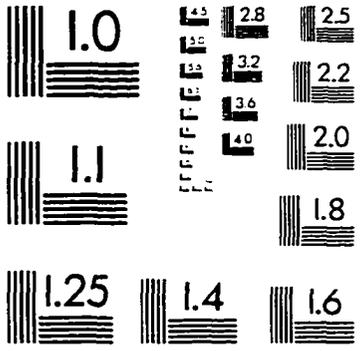

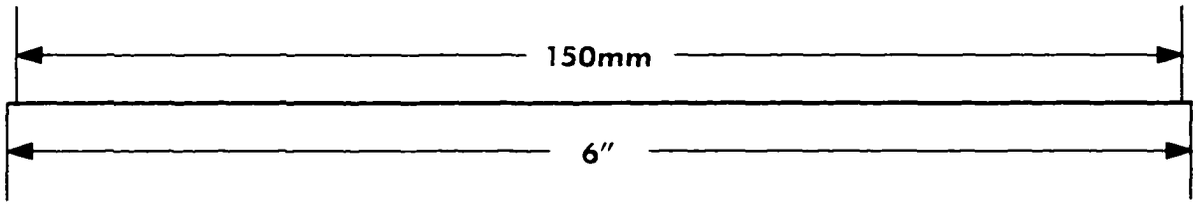

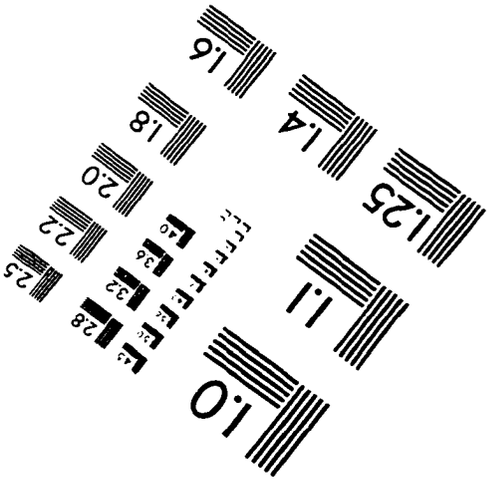

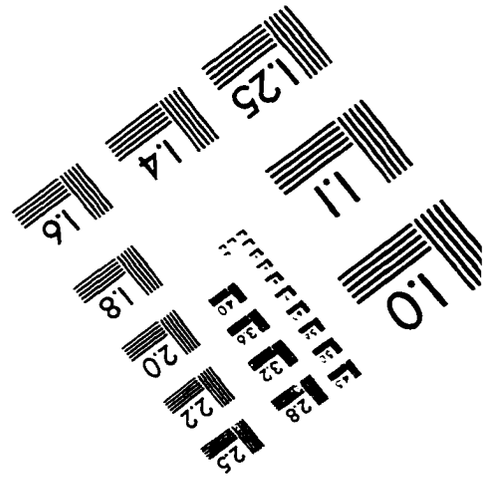

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1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989

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