Hormonal Control of Motor-Terminal Remodeling during Metamorphosis of the Moth, *Manduca sexta*

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ABSTRACT

The tobacco hawkmoth, *Manduca sexta* goes through a complete metamorphosis with a distinct larval, pupal, and adult stage. *Manduca's* metamorphosis is controlled by the insect steroid hormone ecdysone. During this transformation, the neuromuscular system undergoes a hormonally-mediated remodeling that leads to the elimination of synaptic connections required for larval behavior and the development of synapses required for adult behavior. One identified motoneuron, the femoral depressor motoneuron (FeDe MN), is remodeled to innervate a new adult leg muscle after its larval target muscle dies. In the central nervous system (CNS), the FeDe MN's dendrites regress at the end of the larval stage, and new adult dendrites grow during the pupal stage. In the periphery, the FeDe MN's larval target muscle dies at the end of larval life and is replaced by a new adult muscle during the pupal stage. In this thesis, I have asked what controls remodeling of the FeDe MN's terminals in the periphery during the degeneration and regrowth of its target muscle. I have found that the FeDe motor terminals retract during the prepupal stage and subsequently elongate during the pupal stage. In addition, two isoforms of the cell adhesion molecule, Fasciclin II, are expressed by the FeDe MN during motor-terminal remodeling. My results indicate that the ecdysteroids control remodeling of the FeDe motor terminals indirectly through their action on peripheral tissues. Finally, I found that the ecdysteroids regulate expression of Fasciclin II, which is a molecule that has the potential to mediate local cell-cell interactions between the motor terminals and peripheral tissues.

INTRODUCTION

Hormonally-Sensitive Neural Systems

Steroid hormones can exert profound effects on the structure and function of neurons during embryonic development and throughout adult life, which in turn can affect behavior. These effects are most obvious when comparing male and female neuroanatomy. For example, the spinal nucleus of the bulbocavernosus (SNB) innervates the bulbocavernosus and levator ani muscles (BC/LA), which control penile reflexes in rats. Both males and females have a SNB and BC/LA, but they are larger in males than in females. Testosterone has effects on the mass of the BC/LA muscles (Rand and Breedlove, 1992), the dendritic extent of the SNB motoneurons (MNs; Rand and Breedlove, 1995), soma diameter of the MNs (Watson et al., 2001), and the amount of synaptic input that the MNs receive (Leedy et al., 1987).

Beyond their organizational role in establishing the differences in male and female neuroanatomy, steroids can influence the postembryonic plasticity of neurons. For example, seasonal variations in testosterone levels cause fluctuations of the SNB MN's dendritic extent, their soma size, and the mass of the BC/LA muscles over the breeding season (Forger and Breedlove, 1987). Similarly, in the hippocampus of rats, fluctuation in the estrogen level over the 4 day estrous cycle increases the density of dendritic spines on CA1 pyramidal cells (Gould et al., 1990; Woolley et al., 1990), and many of these new spines represent synapses with new postsynaptic cells (Yankova et al., 2001). In addition, estrogen treatment improves learning and memory performance in ovariectamized rats (Daniel et al., 1997). This influence of steroids on neuronal plasticity has many potential clinical applications. In humans, for example, Alzheimer's disease is

more prevalent in women, and estrogen replacement therapy has been implicated in preventing the onset of the defects in memory characteristic of the disease (reviewed by Sherwin, 1999). How and where steroids might act to protect against such memory defects is unknown. The complexity of the vertebrate nervous system often makes it difficult to determine the mode of action of steroid hormones in mediating their multiple effects. We have much to learn about the many effects and mechanisms of steroid hormones. By focusing on a simpler model system, I have contributed to a broader understanding of hormonal actions on the nervous system.

A dramatic example of steroid-induced changes in MN morphology occurs during insect metamorphosis. Identified insect MNs undergo steroid-mediated dendritic regression (Weeks and Truman, 1985), dendritic growth (Levine, 1985; Weeks and Ernst-Utzschneider, 1989) and remodeling of their motor terminals (Truman and Reiss, 1995). The insect nervous system is simple in comparison to the vertebrate nervous system. The MNs are large and uniquely identifiable such that it is possible to work with single identified MNs *in vivo*. Because of this, dendrites and motor terminals of the same MN can be examined to determine if the morphology at both ends is controlled by different signals. In the studies discussed here, I used the tobacco hawkmoth, *Manduca sexta*, to ask if steroid hormones control the developmental plasticity of MNs via direct actions or indirectly via local cell-cell interactions.

Hormonal Control of Insect Metamorphosis

Growth and metamorphosis of *Manduca sexta* are controlled by the steroid hormone ecdysone (reviewed by Riddiford, 1985). Synthesis of ecdysone is preceded by the release of the neuropeptide prothoracicotropic hormone (PTTH) from neurosecretory

cells in the brain (O'Brien et al., 1988; Westbrook et al., 1993), and PTTH then acts on the prothoracic glands to stimulate ecdysone synthesis (reviewed by Gilbert et al., 1996). The prothoracic glands actually secrete 3-dehydroecdysone, which is then converted to ecdysone by enzymes in the hemolymph (reviewed by Nijhout, 1994). Ecdysone is converted to the physiologically active form, 20-hydroxyecdysone (20E), in the mitochondria of the fat body and epidermal cells (Nijhout, 1994). Many naturally occurring analogs and metabolites of ecdysone exist, and because hemolymph ecdysone levels are quantitated with assays that utilize cross-reacting antibodies, it is customary to refer to ecdysone titers as representing "the ecdysteroid" concentration (Nijhout, 1994).

The ecdysteroid titers have been determined throughout *Manduca's* development, and fluctuations in the titer trigger the five larval molts, the pupal molt, and adult development (Figure 1: Bollenbacher et al., 1981). The nature of the molt initiated by the ecdysteroids is determined by Juvenile Hormone (JH). JH titers are high during larval life, and a rise in the ecdysteroid titer in the presence of JH triggers a larval molt (Nijhout, 1994). During the last larval stage, ecdysteroid titers rise in the absence of JH for the first time, and this "commitment pulse" serves to genetically reprogram the larval cells to a pupal fate (Riddiford, 1996a). The subsequent prepupal peak of ecdysteroids initiates the molt to the pupal stage, and the small amount of JH present during this peak prevents the precocious development of the imaginal discs (Kiguchi and Riddiford, 1978). A final sustained rise in the ecdysteroid titer during the pupal stage directs adult development (Bollenbacher et al., 1981).

Significant progress has been made toward understanding the molecular aspects of ecdysteroid action during metamorphosis. Ecdysone binds to a nuclear hormone

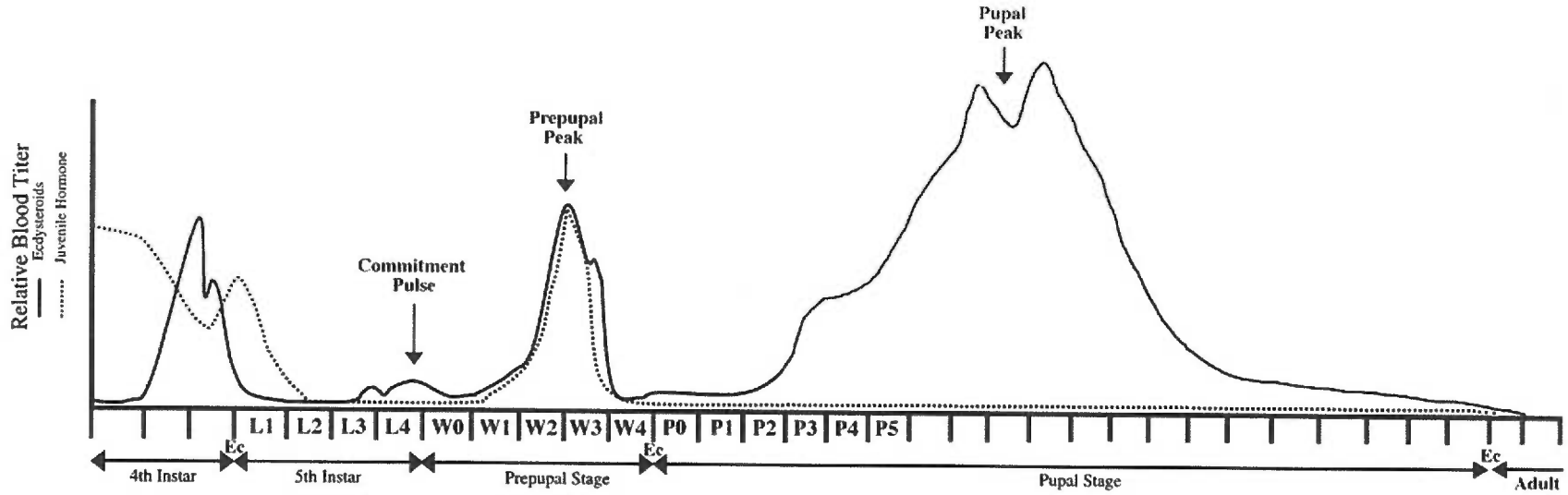

Figure 1. Juvenile hormone (JH) titers are high during larval life (dotted line) and a rise in the ecdysteroid titer (solid line) in the presence of JH triggers a molt to the next laval instar. During the 5th and final larval instar, a rise in the ecdysteroid titer in the absence of JH occurs for the first time in the larva's life. This "commitment pulse" of ecdysteroids genetically reprograms the larval cells to a pupal fate. The subsequent "prepupal peak" of ecdysteroids initiates the molt to the pupal stage. The "pupal peak" of ecdysteroids during the pupal stage leads to adult development. The presence of JH during the commitment pulse or the beginning of the pupal peak can prevent the genetic reprogramming of tissues. Titer adapted from Bollenbacher et al., 1981.

receptor (EcR) that is a member of the steroid receptor superfamily (Koelle et al., 1991). EcR must heterodimerize with Ultraspiracle (USP), which is the insect homologue of the vertebrate retinoid X receptor, before binding to ecdysone response elements (EREs) located on target genes (Yao et al., 1993). In *Drosophila*, the EcR gene encodes three different isoforms of the EcR: EcR-A, EcR-B1, and EcR-B2 (Talbot et al., 1993). Tissues with different metamorphic responses to ecdysone have been shown to express different isoforms of the EcR (Talbot et al., 1993). Larval neurons have no detectable EcR expression during the larval molts but begin to express high levels of EcR-B1 at the onset of metamorphosis (Truman et al., 1994). At the onset of adult development, these larval neurons begin expressing high levels of EcR-A (Truman et al., 1994). Expression of EcR-B1 is associated with the loss of larval features (Schubiger et al., 1998; Truman et al., 1994), while the expression of EcR-A is associated with the development of adult features (Truman et al., 1994). Muscles also express combinations of EcR-B1 and EcR-A during the pupal-adult transformation, and EcR-B1 expression is associated with larval muscle fibers that will be remodeled into new adult muscles (Hegstrom et al., 1998).

The neuronal and muscular genes that are activated as a result of ecdysteroid exposure at the onset of metamorphosis are not well understood. In the larval epidermis of *Manduca*, 20E exposure leads to the expression of the Broad-Complex gene (BR-C; Zhou et al., 1998), which is one of the first genes induced by ecdysone at the onset of metamorphosis and encodes a family of transcription factors (Kiss et al., 1988). Transcripts of BR-C isoforms increase throughout the late larval CNS of *Drosophila* at the onset of metamorphosis, suggesting that MN's response to ecdysone may be similar to that of the epidermis (Restifo and Hauglum, 1998). However, remodeling of

mushroom body neurons in the *Drosophila* brain does not require BR-C, although it does require EcR-B1 and USP (Lee et al., 2000). The genes activated by EcR/USP in MNs and muscles in *Manduca* are unknown.

Juvenile hormone plays an important role during metamorphosis in modulating the tissue's response to the ecdysteroids. JH is capable of preventing the genetic switching that occurs during the larval-to-pupal and pupal-to-adult transitions (reviewed by Riddiford, 1996b). Exposure of a tissue to JH during the commitment pulse prevents the genetic reprogramming of that tissue to a pupal fate. Exposure of MNs and muscles to JH at the beginning of the pupal peak of ecdysteroids has been shown to prevent their adult differentiation (Truman and Reiss, 1988; Truman and Reiss, 1995). The molecular mechanisms by which juvenile hormone (JH) modulates the effects of the ecdysteroids are currently unknown. JH is a hydrophobic molecule (a sesquiterpenoid) that is released from the corpus allatum after it is synthesized (reviewed by Gilbert et al., 1996). JH has been assumed to have molecular actions similar to steroid/thyroid hormones, but a receptor for JH has not been identified (reviewed by Riddiford, 1996b). Nevertheless, JH exposure has been shown to have effects on gene expression. Application of JH at the onset of metamorphosis prevents the appearance of EcR in *Manduca's* nervous system (Riddiford, 1996b). In addition, exposure of larval epidermis to JH during pupal commitment prevents the appearance of Broad-Complex mRNA and protein, which is direct evidence that JH can affect the normal pattern of 20E-induced transcription at the onset of metamorphosis (Zhou et al., 1998; Zhou and Riddiford, 2001).

Remodeling of Neuromuscular Systems during Insect Metamorphosis

Insect metamorphosis illustrates the neural plasticity that steroid hormones can

initiate. The metamorphic remodeling during the transition from a larva to an adult moth requires the nervous system to be dynamic. The nervous system must continue to elicit pupal-specific behaviors while eliminating those neurons specific for larval behavior and adding those specific for adult behavior. Metamorphic restructuring of the nervous system is accomplished through a combination of programmed cell death (Truman, 1983; Weeks and Truman, 1985), neurogenesis (Booker and Truman, 1987a), and respecification of larval neurons for use in adult circuits (Kent and Levine, 1988a; Levine, 1985). These phenomena have all been shown to be controlled by the ecdysteroids (Booker and Truman, 1987b; Truman and Reiss, 1988; Truman and Schwartz, 1984; Weeks and Truman, 1986). The simplicity of the insect nervous system has made it an excellent model for understanding how the ecdysteroids control postembryonic remodeling of individual neurons.

The rising and falling phase of an ecdysteroid titer can cause distinct metamorphic restructuring events. During the remodeling of the larval abdominal muscle DEO1 into adult muscle DE5, the rise and fall of the prepupal peak of ecdysteroids is responsible for the loss of the muscle's contractile apparatus, while the rise in the subsequent pupal peak of ecdysteroids is responsible for breakdown of the larval nuclei (Hegstrom, 1996a). The fall in the pupal peak of ecdysteroids just before adult emergence is responsible for the programmed cell death of this same muscle in the moth (Schwartz and Truman, 1983). Low and high concentrations of 20E can also cause distinct remodeling events. Low concentrations of 20E seem to be responsible for cell proliferation in the developing eye and in the ventral diaphragm muscles, while high concentrations of 20E trigger the arrest of proliferation and lead to the maturation of these tissues (Champlin et al., 1999;

Champlin and Truman, 1998).

Current evidence suggests that 20E induces metamorphic changes by acting directly on the MN's soma to influence gene transcription. Leg MNs maintained in cell culture with 20E have more higher-order branches than those cultured without 20E (Prugh, 1992) and more complex growth cone structure (Matheson and Levine, 1999) suggesting that 20E may induce transcription of genes involved in process outgrowth and/or branching. In addition, abdominal MNs of premetamorphic larvae accumulate radiolabeled 20E analogs (Fahrbach, 1989) and also bind antibodies directed against ecdysone receptor isoforms (Truman et al., 1994), indicating that MNs are equipped to respond directly to the steroid.

Cell-cell interactions are involved in shaping the details of steroid-induced process outgrowth. Ablating the larval leg during *Manduca's* development leads to the growth of inappropriate adult dendrites on the adult FeDe MN (Kent and Levine, 1993). Muscle ablation studies during *Drosophila's* metamorphosis indicate that the formation of secondary motor-terminal branches is delayed until a muscle fiber forms, and delayed muscle development is associated with precocious dendritic arbor development (Fernandes, 1998). Finally, interactions with target muscle seem to control the rate of growth of MN growth cones (Bayline et al., 2001) and the formation of transverse sprouts and the differentiation of synaptic endplates (Truman and Reiss, 1995).

The respective roles played by the ecdysteroids and cell-cell interactions in directing postembryonic remodeling of several abdominal MNs has been investigated. The MNs that innervate the larval prolegs undergo an ecdysteroid-mediated dendritic regression and subsequent programmed cell death as the proleg muscles die at the end of

larval life (Weeks and Truman, 1985). Experiments using heterochronic mosaics indicated that dendritic regression is due to a direct effect of the ecdysteroids on the CNS rather than to the loss of larval sensory inputs during metamorphosis (Jacobs, 1990). In addition, removal of target muscle had no effect on the dendrites, indicating that interactions with target muscle alone were neither sufficient nor necessary for dendritic regression (Weeks and Truman, 1985). Several remodeled MNs that innervate abdominal body wall muscles have also been well characterized. After their larval target muscles die, these MNs are respecified to innervate new adult muscles. In the case of MN-12, retraction of larval motor terminals is controlled by local interactions with the degenerating target muscle (Hegstrom, 1996b), which itself is a direct target of the ecdysteroids (Hegstrom et al., 1998). Elongation of adult motor terminals across the new adult muscle during the pupal stage seems to be controlled by ecdysteroid action on the CNS, while the formation of transverse sprouts and the differentiation of synaptic endplates is controlled by local cell-cell interactions with the target muscle (Truman and Reiss, 1995). Motor-terminal retraction following the programmed cell death of the adult muscle after emergence of the moth appears to be regulated differently. In the adult, local cues do not seem to trigger the loss of adult boutons, since boutons are detectable after the adult muscle degenerates (DeLorme and Mesce, 1999).

One well-characterized respecified MN that I use as a model is the femoral depressor motoneuron (FeDe MN). This MN innervates a femoral depressor muscle in the larva and the adult and is remodeled both centrally and peripherally during metamorphosis. At the end of the larval stage, the larval dendrites regress, and new adult dendrites begin to grow during the pupal stage (Kent and Levine, 1993). Preliminary

data indicated that the larval FeDe muscle is histolysed and the FeDe motor terminals retract just before pupation, and adult FeDe muscle formation and motor-terminal elongation subsequently occur during the pupal stage. (Kent and Bollermann, 1995). Loss of the MN's larval specializations coincides with the prepupal peak of ecdysteroids, and the addition of its adult specializations coincides with the pupal peak (Kent and Bollermann, 1995). I have used this identified MN to ask if motor-terminal remodeling is controlled by a direct action of the ecdysteroids on the soma or by cell-cell interactions between the motor terminals and the muscle by examining both the dendrites and the motor terminals from the same MN.

Nerve-Muscle Interactions During Metamorphic Remodeling

During formation of the larval NMJ, the presence of the nerve is not required for muscle formation. In *Drosophila*, larval muscles are able to form in a mutant that has delayed innervation (Broadie and Bate, 1993b). Uninnervated larval muscle is able to restrict expression of Fasciclin III to synaptic sites and express glutamate receptors similar to innervated muscles, but the presence of the nerve is required to cluster the glutamate receptors to synaptic sites (Broadie and Bate, 1993a). In contrast, development of adult muscles requires the presence of innervation (Kopéc, 1917). During the postembryonic remodeling of respecified NMJs, the nerve retains contact with the muscle and continues synaptic vesicle recycling during the entire process (Consoulas and Levine, 1998), and the presence of the nerve during muscle remodeling allows the nerve to influence adult muscle formation. Adult leg muscles fail to develop in moths that had their leg nerves axotomized during the larval stage (Consoulas and Levine, 1997), suggesting that nerves may play a role in the accumulation and/or proliferation of

myocytes during metamorphosis. Experiments *in vitro* confirm that this effect is due to a role of the nerve in promoting the proliferation of myocytes (Luedeman et al., 1996). In addition, both innervation and 20E exposure are required for the normal upregulation of EcR-B1 receptor expression in muscle (Hegstrom et al., 1998). Therefore, the nerve has many influences on development of adult muscles during metamorphosis.

The intimate association between motor terminals and muscle during the remodeling of respecified MNs allows the muscle to have effects on the MN as well. Studies on respecified abdominal MNs indicate that local interactions between motor terminals and target muscle control motor-terminal retraction (Hegstrom, 1996b). Those studies did not definitively determine if interactions with the target muscle were sufficient for motor-terminal retraction, nor did they investigate the respective roles played by the commitment pulse and prepupal peak of ecdysteroids. During motor-terminal growth, local interactions with the muscle have been shown to control the rate of growth of the MN growth cone (Bayline et al., 2001), but the authors did not determine if this was independent of the MN soma's ecdysteroid exposure. Other studies indicated that interactions with the target muscle could influence the formation of transverse sprouts and synaptic endplates, but that the elongation of motor-terminals across the muscle was controlled by the MN soma's ecdysteroid exposure (Truman and Reiss, 1995). Those experiments, however, were unable to determine if peripheral interactions were sufficient for synaptic endplate differentiation, nor did they address the possibility that peripheral interactions might also be sufficient for elongation.

Role of Cell Adhesion Molecules in Neuromuscular Junction Development

Cell adhesion molecules (CAMs) play a significant role in guiding motor axons to their correct target muscle and organizing the structure of the NMJ (reviewed by Goodman, 1996; Sanes and Lichtman, 1999). In vertebrates, the neural cell adhesion molecule (NCAM) is expressed by motor axons and by muscles during embryonic development (Covault and Sanes, 1986). Once a MN reaches its target and forms a synapse with its target muscle, expression of NCAM becomes restricted to the NMJ (Covault and Sanes, 1986). Axotomy of the motor nerve causes NCAM expression to be upregulated on the MN and the muscle, and once reinnervation occurs, NCAM expression again becomes restricted to the synapse (Covault and Sanes, 1985). NCAM exists as several isoforms that are either transmembrane proteins (TM) or linked to the outer leaflet of the cell membrane through a glycosylphosphatidyl-inositol (GPI) linkage (reviewed by Doherty et al., 1995).

An invertebrate orthologue of NCAM is fasciclin II (Fas II), which has been shown to mediate axon fasciculation and growth cone guidance (Grenningloh et al., 1991; Harrelson and Goodman, 1988) and synaptic stabilization and growth (Schuster, 1996a). A TM isoform of Fas II is expressed on motor nerves and muscles during embryonic development and becomes restricted to the NMJ once synapse formation occurs (Schuster, 1996a). Fas II is required both pre- and postsynaptically for normal NMJ formation, and the relative levels of Fas II regulate synaptic growth. Mutations that decrease Fas II levels to 50% of normal cause NMJs to have excessive synaptic growth, and mutations that decrease Fas II levels to 10% of normal decrease synaptic growth (Schuster, 1996a). In the absence of Fas II expression, active zones form but fail to

sprout and elaborate more boutons, and eventually the nerves retract all together (Schuster, 1996a). Therefore, while a certain level of Fas II during the larval stage is required for synaptic growth, downregulation is actually permissive for synaptic growth. A role for Fas II during metamorphic synapse remodeling has not been examined.

TM and a GPI-linked isoforms of Fas II have been identified in *Manduca sexta* (MFas II; Wright et al., 1999). The expression patterns of these two isoforms of MFas II have been studied in the neurons and glial cells at *Manduca's* enteric plexus. The neurons predominately express TM-MFas II, while the glial cells predominately express GPI-MFas II (Wright and Copenhaver, 2000). During embryogenesis, the cells of the developing peripheral nervous system also express the two isoforms of MFas II in a cell-restricted pattern. The axons and growth cones of nerves express the TM isoform, while the glial cells that ensheath the peripheral nerves express the GPI-linked isoform (Wright and Copenhaver, 2001). Although a GPI-linked isoform of Fas II has been identified in *Drosophila* (Grenningloh et al., 1991), its expression pattern has not been described. In addition, the expression pattern of the two isoforms of MFas II have not been examined during the postembryonic remodeling of *Manduca's* neuromuscular system.

Questions Addressed in this Thesis

The overall goal of my thesis is to determine the respective roles played by hormones and cell-cell interactions in remodeling of the FeDe MN's motor terminals during metamorphosis. While remodeling of the FeDe MN's motor terminals occurs during a time that the ecdysteroid titers are fluctuating, and the ecdysteroids seem to be responsible for the changes in dendritic morphology, it is not clear if motor-terminal remodeling is caused by direct or indirect actions of the ecdysteroids on the FeDe MN.

First, I asked what the time course of postembryonic motor-terminal remodeling was and whether or not MFas II was expressed at the FeDe NMJ during this process. Second, I investigated the hormonal control of retraction of FeDe motor terminals at the end of larval life. Third, I investigated the hormonal control of growth of FeDe motor terminals during adult development. Finally, I asked if the ecdysteroids regulate the expression of TM-MFas II by the FeDe MN during motor-terminal retraction.

CHAPTER 1

Remodeling of motor terminals during metamorphosis of the moth *Manduca sexta*: expression patterns of two distinct isoforms of *Manduca* fasciclin II

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SUMMARY

During metamorphosis of the moth *Manduca sexta*, the neuromuscular system of the thoracic legs is reorganized dramatically. Larval leg muscles degenerate at the end of larval life, and new adult leg muscles develop during the ensuing pupal stage. Larval leg motoneurons persist, but undergo substantial remodeling of central and peripheral processes. As part of our on-going investigation of mechanisms underlying the remodeling of motor terminals, we have used antisera generated against *Manduca*-specific isoforms of the homophilic adhesion molecule fasciclin II (MFas II) to label motor terminals during metamorphosis. Antisera generated against the GPI-linked isoform of MFas II (GPI-MFas II) labeled the motor nerves at all stages and appeared to be associated with glial cells ensheathing the peripheral nerves. In addition, the anti-GPI-MFas II antisera labeled regions associated with synaptic boutons at both larval and adult stages. In contrast, antisera generated against a transmembrane isoform of MFas II (TM-MFas II) only labeled specific neuronal processes at discrete intervals during remodeling. Identified leg motoneurons (such as the femoral depressor motoneuron) expressed detectable levels of TM-MFas II in their peripheral processes only during phases of motor-terminal retraction and initial stages of motor-terminal re-growth. Putative modulatory neurons (such as the unpaired median neurons), however, expressed TM-MFas II in their processes during larval stages as well as during remodeling. Use of the isoform-specific anti-MFas II antisera provided a novel method for visualizing remodeling of motor terminals during metamorphosis and helped distinguish different components of the motor nerves and neuromuscular junction.

INTRODUCTION

The neuromuscular junction displays structural and functional plasticity throughout an animal's life, such that synaptic endplates may be eliminated, regenerated, or remodeled. Although it is clear that an activity-dependent competition between axons is involved in synapse elimination, the factors mediating the events are still unknown (reviewed by Lichtman and Colman, 2000; Sanes and Lichtman, 1999). Similarly, multiple signals associated with terminal Schwann cells, the synaptic basal lamina, and perisynaptic interstitial cells are involved in the process of re-innervation, but the identity of these signals and their respective roles are still under investigation (Ide, 1996; Son and Thompson, 1995; Terenghi, 1999).

Invertebrates also exhibit postembryonic remodeling of the neuromuscular junction. A particularly dramatic example occurs during metamorphosis of holometabolous insects. The larval target muscles of many motoneurons die during metamorphosis while the motoneurons persist. The persistent motoneurons are respecified to innervate new adult target muscles and undergo remodeling of their central and peripheral processes (reviewed by Tissot and Stocker, 2000). In the periphery, a period of motor-terminal retraction and loss of synaptic boutons is followed by a period of motor-terminal sprouting and the differentiation of a new complement of synaptic boutons. This phenomenon has been particularly well characterized during the metamorphosis of the tobacco hornworm, *Manduca sexta*, when the muscles of the larval thoracic legs degenerate and new adult leg muscles are formed (Consoulas et al., 1997; Kent et al., 1995). The leg motoneurons, in contrast, survive and are remodeled to

innervate the adult leg muscles (Consoulas et al., 1996; Kent and Levine, 1988a; Kent and Levine, 1993).

Respecification of abdominal motoneurons during metamorphosis has been shown to be controlled by the insect steroid hormone, 20-Hydroxyecdysone (20-HE; Weeks and Levine, 1990). Specific aspects of motor-terminal remodeling, however, may be influenced by cues derived from target muscle (Hegstrom, 1996b; Truman and Reiss, 1995). Although the nature of these cues remains unknown, several molecules have been identified in *Drosophila* that mediate cell-cell interactions during the formation of the neuromuscular junction. Among these, the homophilic adhesion molecule fasciclin II (Fas II) has been shown to regulate synaptic growth during development of the larval neuromuscular junction (Schuster, 1996a). During embryogenesis, Fas II is initially expressed on all motor axons and their growth cones (Van Vactor et al., 1993) and across the surface of muscles (Schuster, 1996a). After the formation of the mature neuromuscular junction, however, expression of Fas II in both motoneurons and muscles becomes localized to the synapse. A critical level of Fas II on the motoneuron and on the muscle is required for synaptic stabilization and growth (Schuster, 1996a), and a downregulation of synaptic Fas II is involved in synaptic growth and plasticity (Schuster et al., 1996b). A role for Fas II during metamorphic motor-terminal remodeling, however, has not yet been investigated.

Two Fas II isoforms have been cloned from *Manduca* (MFas II). One isoform (~95kDa) spans the cell membrane and has an intracellular carboxy-terminus (TM-MFas II), while the other isoform (~90kDa) is attached to the outer leaflet of the cell membrane via a glycosyl-phosphatidylinositol anchor (GPI-MFas II; Wright et al., 1999). In the

embryonic enteric nervous system of *Manduca*, these two isoforms are expressed in cell and stage-specific patterns. For example, a migratory population of neurons (EP cells) express the TM isoform of MFas II during their periods of active migration and axon outgrowth, while the glial cells that subsequently ensheath the EP cells express the GPI-linked isoform (Wright and Copenhaver, 2000). Expression of MFas II is required for normal migration and each isoform appears to play a distinct role (Wright and Copenhaver, 2000; Wright et al., 1999).

As a prelude to investigating a role for MFas II during motor terminal remodeling, we have used isoform-specific antisera to describe the patterns of expression of the two isoforms of MFas II associated with the femoral depressor (FeDe) muscles of the metathoracic leg. The FeDe muscles are innervated by an identified motoneuron, the FeDe motoneuron, and by a putative modulatory neuron known as an unpaired median (UM) neuron. The FeDe motoneuron innervates the larval leg muscles via processes that branch along their length to form endplates with a rosette-like pattern (type I terminals; Consoulas et al., 1999). Our results indicate that TM-MFas II is expressed on the somata and processes of the FeDe motoneuron only during prepupal and early pupal stages, coincident with the metamorphic remodeling of the leg motoneurons. UM neurons, however, innervate larval leg muscles via thinner processes that have varicosities along their length (type II terminals; Consoulas et al., 1999), and express TM-MFas II uniformly on their somata and processes during larval and early pupal stages. In contrast, the GPI-linked isoform of MFas II appears to be expressed by glial cells that ensheath the axons and processes of motor and modulatory neurons throughout postembryonic life. GPI-MFas II also appears to be localized to regions associated with type I synaptic

boutons, a finding that might reflect differences in the organization of the neuromuscular junction of *Manduca* and *Drosophila*. Although a GPI-linked isoform of Fas II is present in *Drosophila* (Grenningloh et al., 1991; Lin et al., 1994), only TM-Fas II has been described at mature larval synaptic boutons (Schuster, 1996a).

Additional studies will be required to address the roles played by these two distinct isoforms of MFas II at the neuromuscular junction during postembryonic development. Nevertheless, expression of the TM-MFas II isoform on motor terminals during metamorphic remodeling suggests that TM-MFas II may play a role during postembryonic development of the neuromuscular junction in *Manduca*, similar to that played by TM-Fas II during embryonic development of *Drosophila*. The expression pattern of GPI-MFas II at the neuromuscular junction, in contrast, could reflect a novel role for the GPI-linked isoform in *Manduca*. A preliminary account of these results has been reported in abstract form (Knittel et al., 1999; Knittel et al., 2000).

MATERIALS AND METHODS

Animals

Manduca sexta (Lepidoptera:Sphingidae) larvae, pupae, and moths were obtained from a laboratory culture reared on artificial diet (Bell and Joachim, 1976) on a long-day photoperiod regimen (17 hours light, 7 hours dark) at 26° C and approximately 60% relative humidity. Developmental stages were determined using both chronological and morphological criteria (Nijhout and Williams, 1974; Reinecke et al., 1980; Tolbert et al., 1983; Truman and Riddiford, 1974; Truman et al., 1980). For the prepupal stages, W0 signifies the first day of "wandering," and W1, W2, etc., refer to the ensuing prepupal days. W0 stage larvae were identified by the exposure of the dorsal vessel; stage W1 and W2 begin 24 or 48 hours after this point, respectively. We have subdivided W2 into W2a and W2b, which are defined as the periods on day W2 before and after ocellar retraction occurs, as described previously (Consoulas et al., 1996). Animals maintained under our rearing conditions typically pupated on day 4 or 5 after the onset of wandering. Pupal stages are designated P1, P2, etc., with P1 designating the first 24 hours after pupation. Pupae were also staged based on morphological criteria visible through the pupal cuticle ; (personal communication, L.P. Tolbert; Tolbert et al., 1983). Prior to dissection, animals were anesthetized by immersion in ice water.

Biocytin labeling of axons and terminals

To reveal the entire extent of the peripheral branching on the larval FeDe muscle, a biocytin protocol was used (Consoulas et al., 1996). After removing the head and the abdomen, the thoracic segments were dissected along the dorsal midline and pinned down on a Sylgard-coated petri dish in *Manduca* saline (Weevers, 1966). The entire

metathoracic ganglion with intact nerves was isolated from the surrounding saline in a petroleum jelly well that was filled with distilled water. The ganglion was then severed from the leg nerves to allow the infusion of a biocytin solution (3% w/v in distilled water; Sigma). The preparations were stored at 4° C for 2-4 days, subsequently dissected, and fixed overnight in freshly prepared fixative solution (4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4). The preparations were incubated in ABC (Vector Laboratories) overnight, and labeling was revealed by reacting with 3', 3', diaminobenzidine (DAB, Sigma).

Immunohistochemistry

To visualize the motor terminals, we used a *Drosophila* anti-synaptotagmin antibody (anti-Dsyt2; generously provided by J.T. Littleton and H.J. Bellen). Synaptotagmin is an integral membrane protein involved in synaptic vesicle docking (Sudhof and Jahn, 1991) and has been shown to label synaptic boutons in *Drosophila* (Littleton et al., 1993) and in *Manduca* (Consoulas et al., 1996). Following dissection in a zero-calcium saline [modified from (Miyazaki, 1980); J.C. Weeks, personal communication], legs were fixed overnight in 4% paraformaldehyde. Tissue was subsequently rinsed in phosphate buffered saline, (PBS, pH 7.4), permeabilized in PBS + 0.2% triton-X-100 (TX), and incubated 1-24 hours in 10% normal goat serum (NGS) in PBS + TX. Anti-synaptotagmin was used at 1:1000 in 5% NGS in PBS + TX for 24-72 hours. Tissue was subsequently processed for immunohistochemistry using a biotinylated goat-anti-rabbit secondary antibody, an elite ABC kit (Vector), and DAB as the substrate.

To visualize endplate glia, we used a *Manduca* anti-neuroglian antibody (MAb3B11, generously provided by J.B. Nardi). MAb3B11 recognizes *Manduca* neuroglian, which is the insect ortholog of mouse L1, and is expressed in neural and non-neural tissues (Bieber et al., 1989; Chen et al., 1997; Nardi, 1994). At the neuromuscular junction of *Manduca*, MAb3B11 provided an unambiguous marker for endplate glia and labeled a variety of neural and non-neural structures as well.

To label the cells at the neuromuscular junction that express the TM isoform of Fas II, dissections were performed in PBS, and the tissue was fixed in Bouin's solution (Humason, 1979) or a buffered picric acid-formaldehyde solution, pH 7.0-7.5, (Zamboni and Demartino, 1967) at 4° C overnight. Bouin's-fixed tissue was subsequently rinsed for 24-48 hours in 70% ethanol saturated with lithium carbonate to remove the picric acid. After being returned to PBS through a graduated ethanol series, tissue was incubated in 10% NGS for 1-24 hours. Anti-Fas II-TM antisera (anti-TM-MFas II) were used at 1:2000 in PBS + TX and 5% NGS for 24-72 hours. The tissue was processed for immunohistochemistry using a biotinylated goat-anti-guinea-pig secondary antibody, an elite ABC kit, and DAB.

To label the cells at the FeDe neuromuscular junction that express the GPI-linked isoform of Fas II, dissections were performed in PBS and tissue was fixed in 4% paraformaldehyde overnight at 4° C. Tissue was incubated in anti-Fas II-GPI at 1:20,000 in PBS + TX and 5% NGS and processed for immunohistochemistry as described above.

The anti-MFas II antisera used in this study were generated as described by Wright and Copenhaver (2000). Briefly, guinea pigs were immunized with synthetic peptides specific for the intracellular domain of TM-MFas II or the region associated

with the GPI-linkage. Specificity was confirmed by immunoblot analyses (Wright and Copenhaver, 2000). Antisera that had been preadsorbed by incubation with the synthetic peptides were used to verify specificity of labeling in our studies.

Double Labeling-Laser Scanning Confocal Microscopy

Tissue was prepared as described above, but for most preparations an Alexa Fluor 594-conjugated goat-anti-guinea-pig antibody (Molecular Probes) was used to visualize MFas II labeling. An Alexa Fluor 488-conjugated goat-anti-rabbit or goat-anti-mouse antibody (Molecular Probes) was used to visualize synaptotagmin or neuroglian labeling, respectively. In some preparations, propidium iodide (25 μM) was used to label cell nuclei following a 15 minute incubation in RNAse (0.1 mg/mL PBS). Images were acquired with a BioRad MRC 1024 ES laser scanning confocal microscope (CLSM) attached to a Nikon Eclipse TE300 inverted fluorescent microscope fitted with a krypton/argon laser generating excitation lines at 488, 568 and/or 647 nm. Optical sections of 0.5 -1 μm were recorded through the depth of whole-mount preparations.

Image Processing

Whole-mount biocytin and immunostained preparations were drawn at 10x using a *camera lucida* attached to a Zeiss compound microscope. Preparations were photographed at 20x or 40x with a 35mm Contax camera attached to a Zeiss or Nikon compound microscope. Color slides were scanned and images were digitized using a Nikon LS 1000 slide scanner and Adobe Photoshop 4.0 software. Image processing consisted only of adjusting contrast and brightness of the black and white images.

RESULTS

Innervation of the femoral depressor muscles of the metathoracic leg

Anterograde biocytin staining of the entire leg nerve was used to reveal the overall pattern of innervation of the larval FeDe muscles (Fig. 1A). A branch (arrowhead in Fig. 1A) of the main leg nerve, IIIN2a, extended toward the medial FeDe muscles from the point at which IIIN2a passed ventral to the sternotrochanteral muscle (STR; Eaton, 1982), prior to entering more distal leg segments. The branch split into medial and anterior branches, such that the anterior branches extended to reach the more anterior groups of FeDe muscles while the medial branches innervated the medial FeDe muscles. The primary nerve branches split into secondary nerve branches to reach each muscle fiber in each group, and multiple tertiary nerve branches were associated with rosette-like endplates along each muscle fiber (arrow, Fig. 1B). Anti-synaptotagmin labeling also revealed the rosette-like arrangement of synaptic boutons on the larval muscle fibers (arrows, Fig. 1C), as described previously for more distal leg muscles (Consoulas et al., 1996) and designated type I terminals (Consoulas et al., 1999). Unlike the biocytin staining, however, none of the primary, secondary, or tertiary branches of the nerve labeled with anti-synaptotagmin at this stage.

Surprisingly, anti-GPI-MFas II labeled the motor nerve in a pattern similar to that seen with biocytin staining. Thus, the entire leg nerve including higher order branches were labeled (Fig. 2A, B). More lightly stained areas at the tips of the higher-order branches appeared to outline the terminal varicosities (small arrow, Fig. 2B). Double-labeling and confocal microscopy confirmed that anti-synaptotagmin-positive boutons were surrounded by anti-GPI-MFas II-labeled regions (Fig. 3A). Anti-GPI-MFas II did

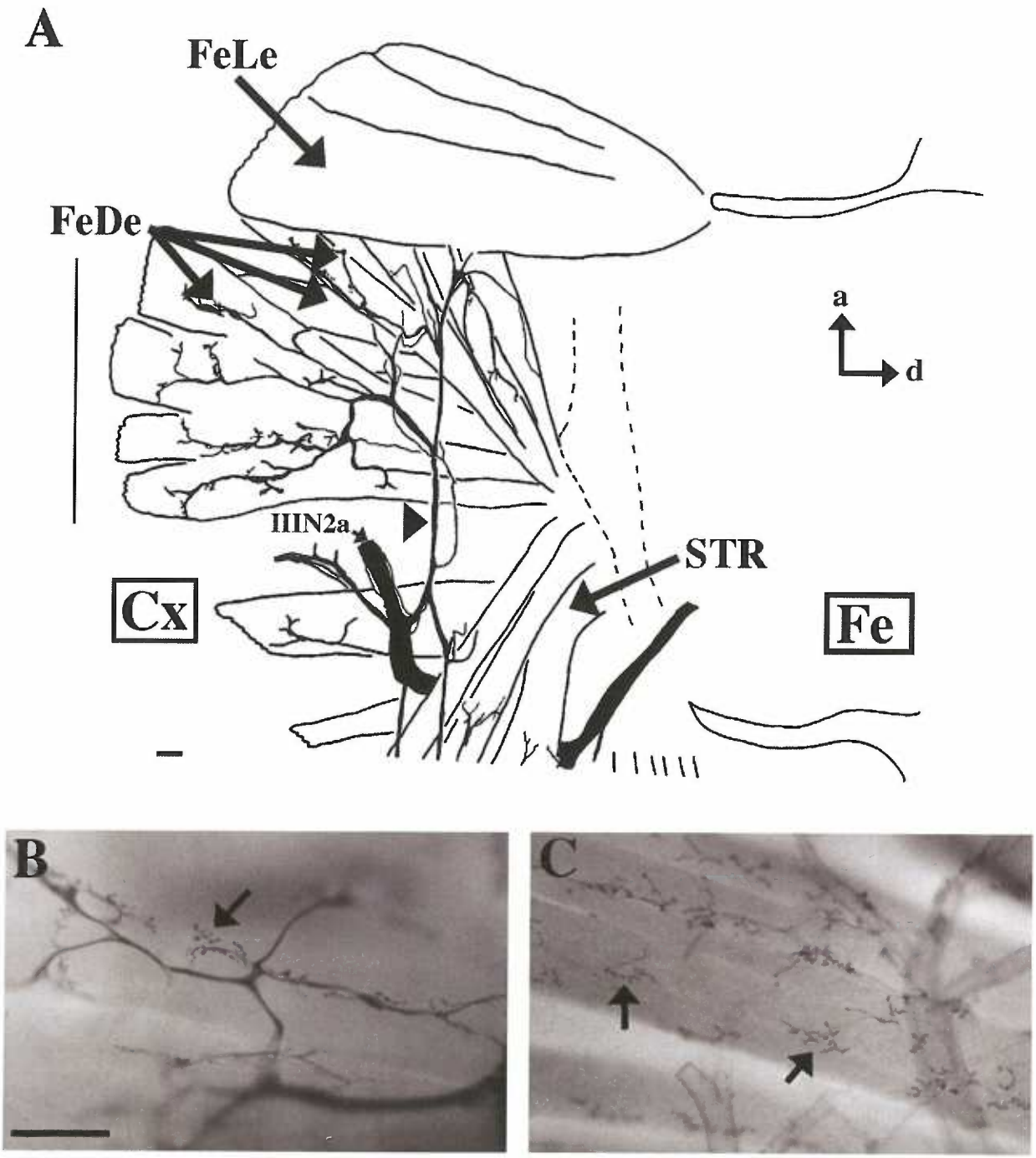

Figure 1. Neuromuscular junction of the FeDe muscle in the fifth larval instar. (A) Camera lucida drawing showing the innervation of the FeDe muscles of the larval metathoracic leg after biocytin staining. Arrowhead indicates the branch of the main leg nerve projecting to the FeDe muscles. All subsequent camera lucida drawings are shown at the same scale and same orientation. (B, C) Photographs of whole-mount FeDe muscles after (B) biocytin staining of the main leg nerve IIIN2a or (C) anti-synaptotagmin immunostaining of synaptic boutons. Arrows in B and C indicate the rosette-like arrangement of the synaptic boutons. Cx, coxa; Fe, femur; STR, sternotrochanteral muscle; FeDe, femoral depressor muscle; FeLe, femoral levator muscle; a, anterior; d, distal. Bars, 100 μm.

not label central neurons, but rather appeared to be associated with glial cells that cover the ganglion (Fig. 2C) and ensheath the peripheral nerves (arrowheads, Fig. 2B). Double-labeling with anti-GPI-MFas II and the nuclear label propidium iodide demonstrated that glial cell bodies associated with the peripheral nerves were labeled with anti-GPI-MFas II (Fig. 3B). Both large and small diameter nerves were labeled with anti-GPI-MFas II and individual nuclei conformed to the diameter of the nerves. An anti-neuroglian antibody (Chen et al., 1997; Nardi, 1994) provided an independent marker for peripheral glia and labeled the peripheral nerves in a pattern that overlapped with anti-GPI-MFas II labeling. At the endplates, however, anti-neuroglian labeling was more broadly distributed around the terminals than anti-GPI-MFas II labeling, indicating that the endplate glial cells formed a covering over the terminals that extended beyond the immediate synaptic region (arrowhead, Fig. 3C). Thus, the anti-GPI-MFas II did not uniformly label endplate glia. Nevertheless, the anti-GPI-MFas II labeling around the boutons could represent a localization of GPI-MFas II to the processes of the endplate glia that interdigitate between sites of nerve-muscle contact.

In contrast to the extensive anti-GPI-MFas II labeling of the peripheral nerves, anti-TM-MFas II labeling was restricted to only a few axons traveling within the ventral nerve. The labeled axons branched and sent thin, straight processes along many or all of the leg muscles, including the FeDe muscle (Fig. 2D, E). Small varicosities along the length of the thin processes (arrow, Fig. 2E) resembled boutons characteristic of UM neurons and designated type II terminals by Consoulas et al. (1999). In the metathoracic ganglion, cell bodies of a small number of neurons were labeled, most notably the UM neurons (arrow, Fig. 2F). The axons arising from these neurons were also labeled and

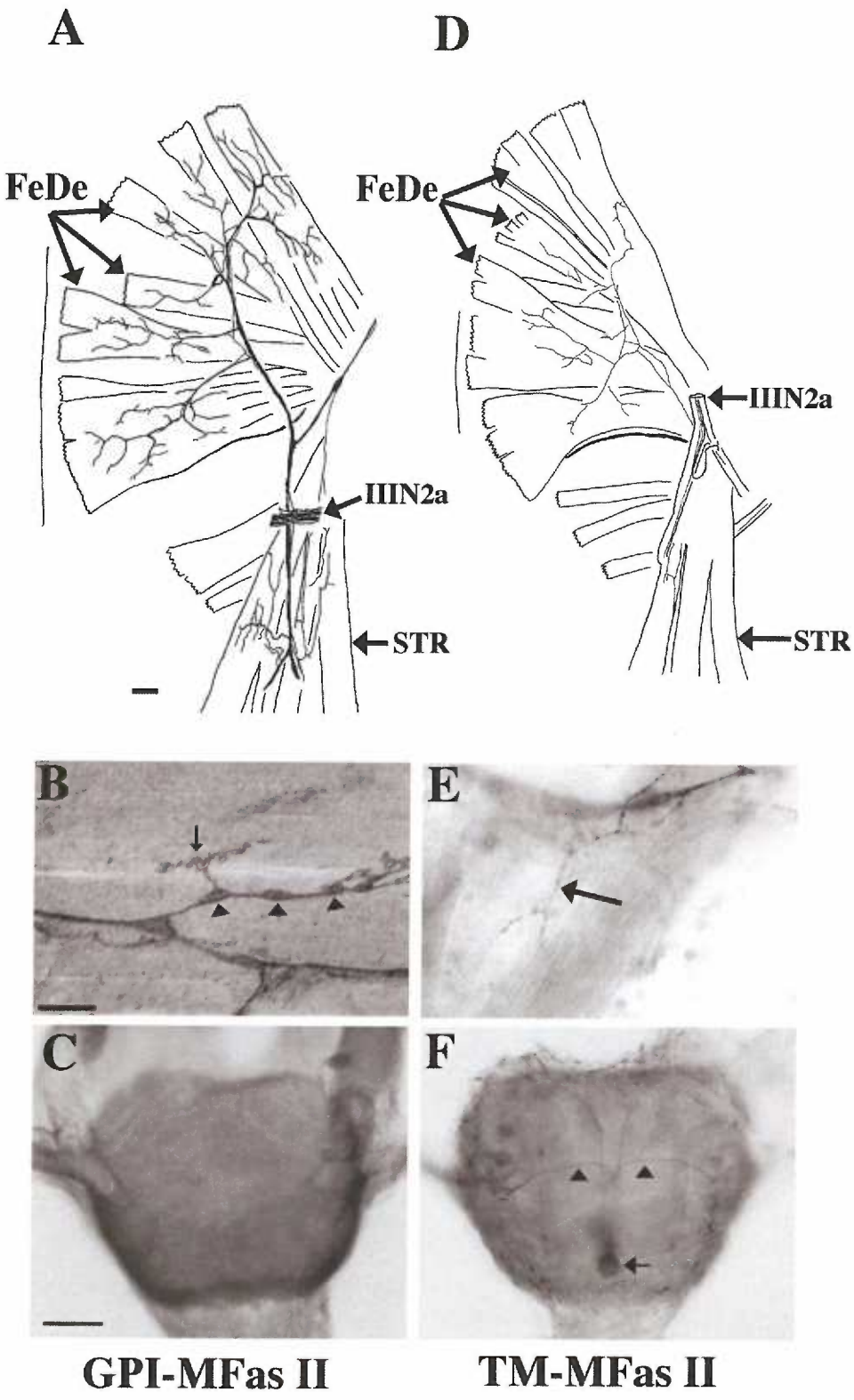

Figure 2

formed the bilateral branches characteristic of UM neurons that projected out the ventral nerves (arrowheads, Fig. 2F). Double-labeling and confocal microscopy indicated that TM-MFas II and synaptotagmin were co-expressed at type II synaptic boutons (arrow, Fig. 3D), as well as along the length of the type II processes. In contrast, TM-MFas II was not detectable in processes associated with type I terminals (arrowhead, Fig. 3D). Moreover, anti-TM-MFas II labeling localized to the type I synaptic boutons, as observed in *Drosophila* (Schuster, 1996a), was difficult to detect in our preparations. In some preparations, however, faint labeling at the type I boutons could not be ruled out (not shown).

Degeneration of the metathoracic FeDe muscles and retraction of motor terminals during prepupal stages

The motor terminals associated with the larval FeDe muscle began to retract during the prepupal stage as the muscle bundles began to show signs of degeneration. On W+2b, muscle fibers were intact but became shortened and condensed in appearance. Anti-GPI-MFas II continued to label the motor branches and terminals during this stage (Fig. 4A). By W+2, many more central neurons were labeled with anti-TM-MFas II (compare Fig. 2F with 4B), including neurons that were in the correct positions to be the previously identified FeDe motoneurons (FeDeMNs; Kent and Levine, 1993); arrows, Fig. 4B). Retrograde labeling of the FeDeMNs by injection of fluorescently tagged lysinated dextran (10,000 MW, Molecular Probes) into the FeDe muscles combined with anti-TM-MFas II labeling confirmed that the anti-TM-MFas II-positive somata located in those positions were indeed those of the FeDeMNs (see Fig. 5E, F). Notably, processes

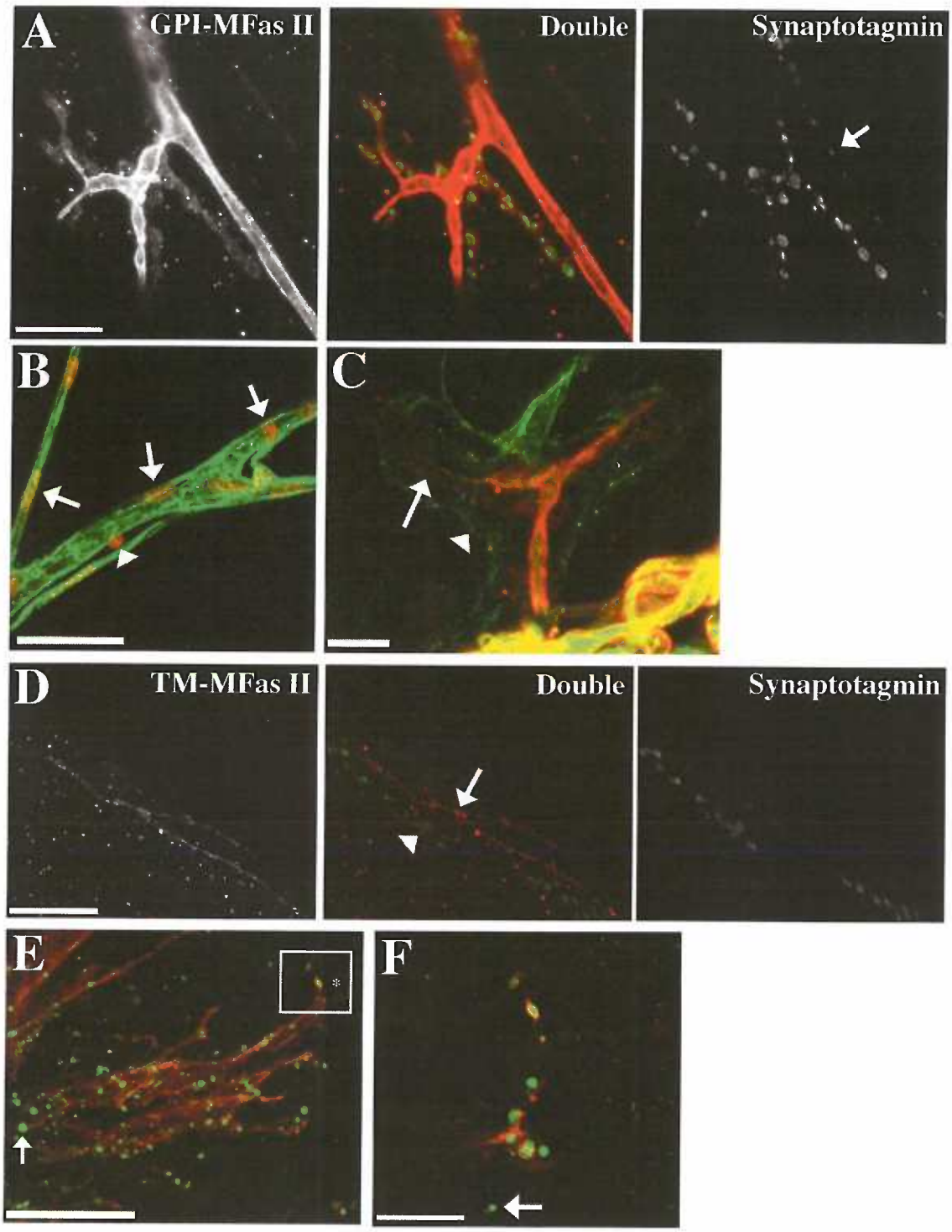
A
GPI-MFas II
Double
Synaptotagmin
B
C
D
TM-MFas II
Double
Synaptotagmin
E
F

Figure 3

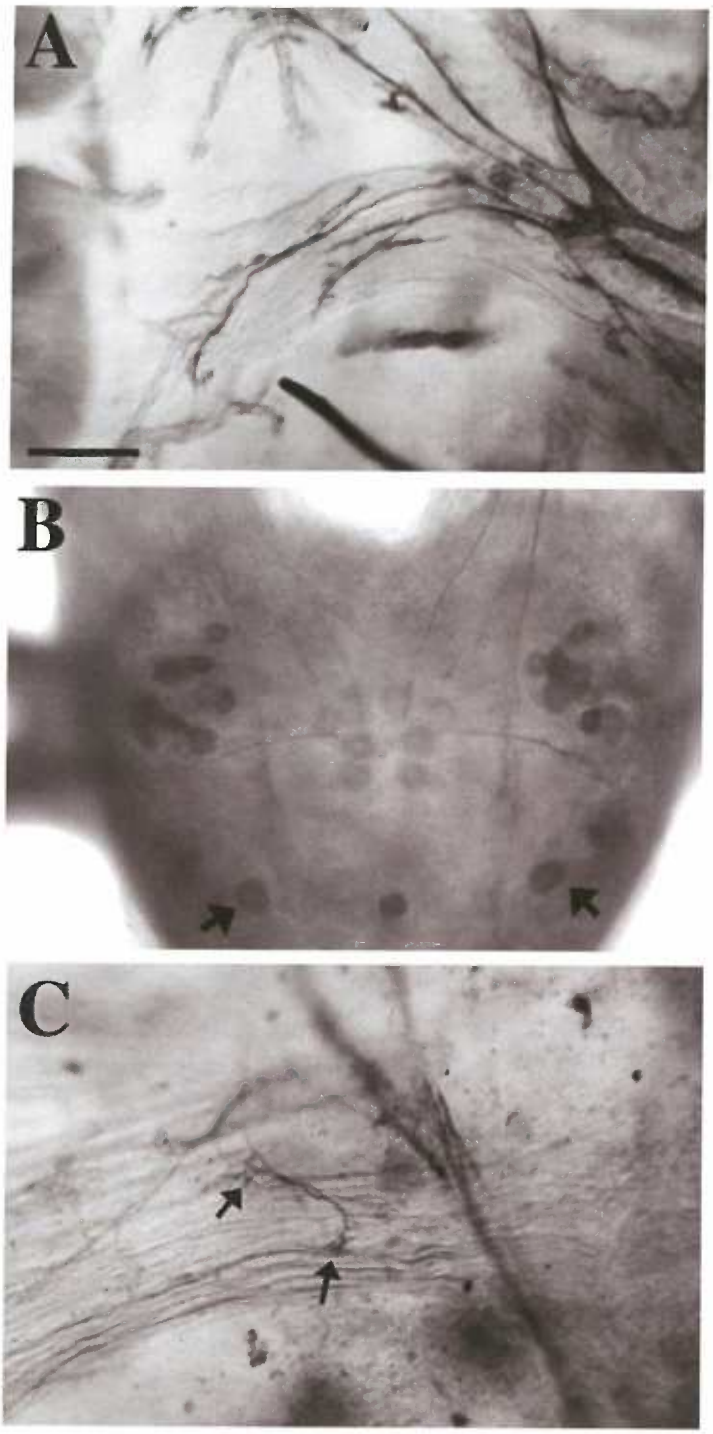

Figure 4. Immunostaining with anti-MFas II antisera as the FeDe muscles undergo degeneration on W+2. (A) Anti-GPI-MFas II continues to label motor branches and areas associated with synaptic boutons on the FeDe muscles. (B) Anti-TM-MFas II labeling of the metathoracic ganglion at this stage reveals many more central neuronal somata, including some in the correct positions to be the FeDe MNs (arrows). (C) Anti-TM-MFas II labeling in the periphery reveals profiles resembling type I terminals associated with the FeDe muscles (arrows). Bar, 100 μm.

resembling type I motor terminals also began to stain positively for TM-MFas II on the FeDe muscles at this stage (arrows, Fig. 4C).

By W+3, all bundles of the FeDe muscle had undergone significant degeneration. Remnants of both anterior and medial muscle groups were still visible, but muscle fibers were thin and wispy in appearance and were difficult to distinguish. Anti-GPI-MFas II labeled thick branches associated with the degenerating muscle (arrows, Fig. 5A, B), but finer branches and synaptic terminals were no longer apparent (Fig. 5A, B). Double-labeling and confocal microscopy revealed what appeared to be remnants of endplates and indicated that many anti-synaptotagmin-immunoreactive varicosities were still associated with anti-GPI-MFas II labeling at this stage (Fig. 3E, F). Other anti-synaptotagmin positive varicosities lacked an obvious association with anti-GPI-MFas II labeled processes (arrow, Fig. 3F), and some were quite large, suggesting that synaptic boutons had coalesced into larger structures (arrow, Fig 3E). On day W+3, anti-TM-MFas II labeled many fine processes in the periphery (small arrows, Fig. 5C, D), some of which extended along the length of the degenerating muscle. At this stage, anti-TM-MFas II also labeled processes that extended from the main nerve branches and formed tuft-like endings (large arrows, Fig. 5D).

Growth of the motor terminals and the development of the adult FeDe muscles during pupal stages

On the day of pupation (P1), the FeDe muscle remnant (the adult muscle anlagen) consisted of a small mass in the anterior-medial region of the developing adult coxa that was associated with the newly developed median tendon of the trochanter (Fig. 6A, C). Anti-GPI-MFas II labeling was associated with both coarse and fine processes (Fig. 6B).

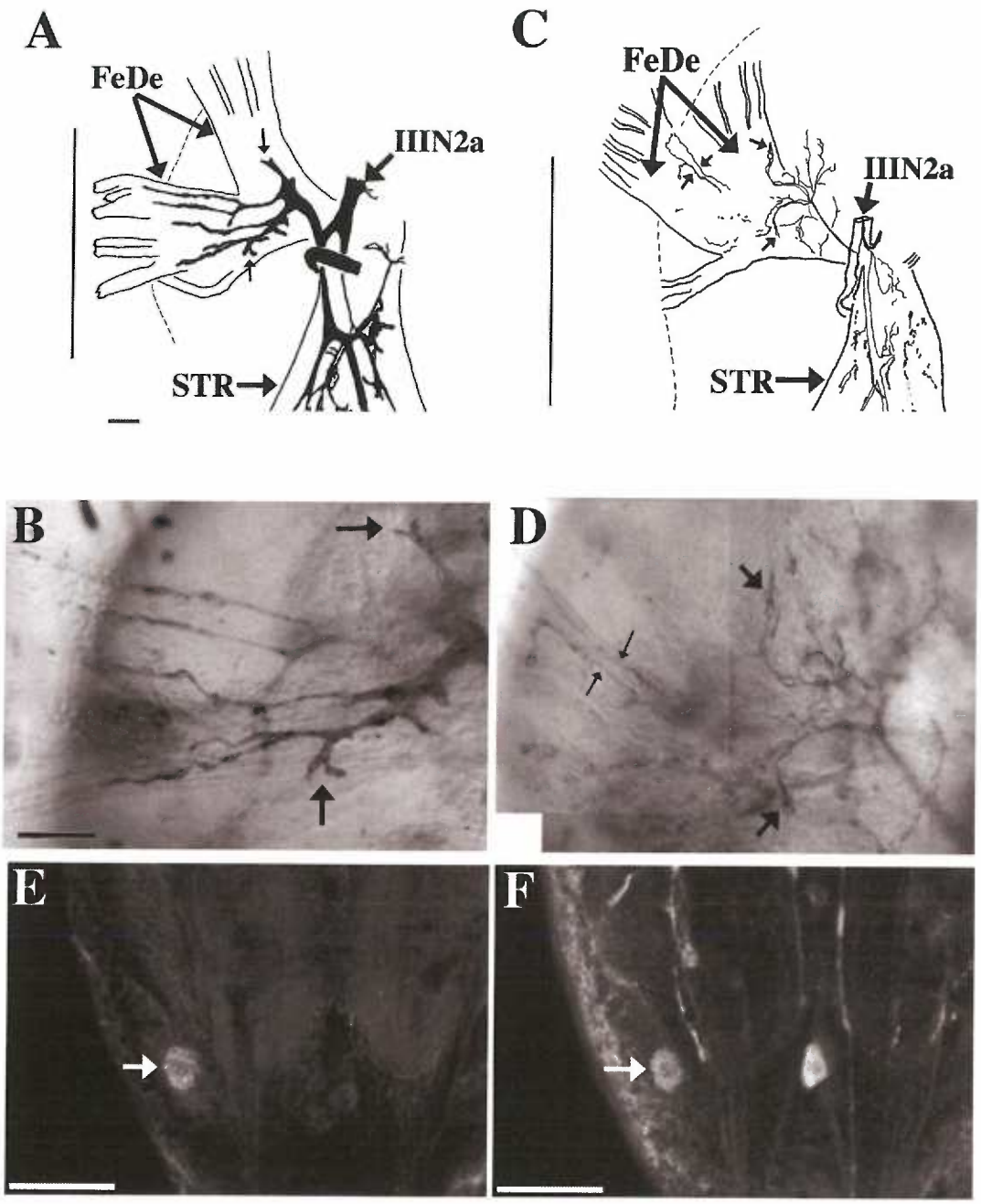

Fig. 5 Anti-MFas II labeling on W+3. (A, C) Camera lucida drawings of anti-MFas II labeling showing thick processes labeled with anti-GPI-MFas II (A) and thin processes labeled with anti-TM-MFas II (C) on the degenerating FeDe muscles. Arrows on camera lucida drawing correspond to arrows in the photographs. Bar, 100 μm. (B, D) Photographs of the whole-mount FeDe muscles drawn in (A) and (C). Bar, 50 μm. (D) is a montage of two photographs taken in different planes of focus to show thin processes associated with remnants of individual muscle fibers (small arrows) as well as tufts that might represent new growth (large arrows). (E, F) Confocal micrographs of the FeDeMN retrogradely labeled from the FeDe muscle with Alexa-Fluor-488 tagged dextran (E) and immunostained with anti-TM-MFas II and visualized with an Alexa-Fluor-594 tagged secondary antibody (F) confirm that the FeDeMN (arrows in E,F) is anti-TM-MFas II positive at this stage. Bars, 100 μm.

Double-labeling and confocal microscopy indicated that there were few anti-synaptotagmin immunoreactive varicosities within the anlagen and most were associated with anti-GPI-MFas II positive processes, though an occasional varicosity was not (arrow, Fig. 9A). Anti-TM-MFas II labeled fine processes that extended along the anlagen, but unlike anti-GPI-MFas II, it did not label thicker processes (Fig. 6C, D).

Although the adult FeDe muscle showed obvious growth during the early pupal stages, additional motor-terminal retraction occurred between stages P2 and P3. Processes that had covered the entire extent of the muscle anlagen at stage P1 and P2 were gone by P3 or P4 (compare Figs. 6C and 7B). Anti-GPI-MFas II labeling at this stage was associated with the nerve branch innervating the central region of the FeDe muscle as well as with fine processes that extended from it (Fig. 7A). The proximal portions of these processes had a scalloped appearance, suggestive of glial cell bodies (arrows, Fig. 7C). Double-labeling and confocal microscopy revealed that anti-synaptotagmin immunoreactivity had increased dramatically by P3, such that many fine neurites were labeled. Most anti-synaptotagmin-immunoreactive neurites were closely associated with anti-GPI-MFas II-immunoreactive processes even at their distal-most tips (Fig. 9B). However, a few tiny neurites, suggestive of filopodia, were not associated with anti-GPI-MFas II positive processes (arrow, Fig. 9B). Anti-TM-MFas II staining at this stage revealed a pattern similar to that observed with anti-GPI-MFas II staining, but without the scalloped appearance. Instead, small varicosities were located along the length and at the tips of the neurites (arrows, Fig. 7D).

Between stages P5 and P7, the FeDe muscle underwent significant growth and differentiation, and both anti-MFas II antisera continued to label processes extending

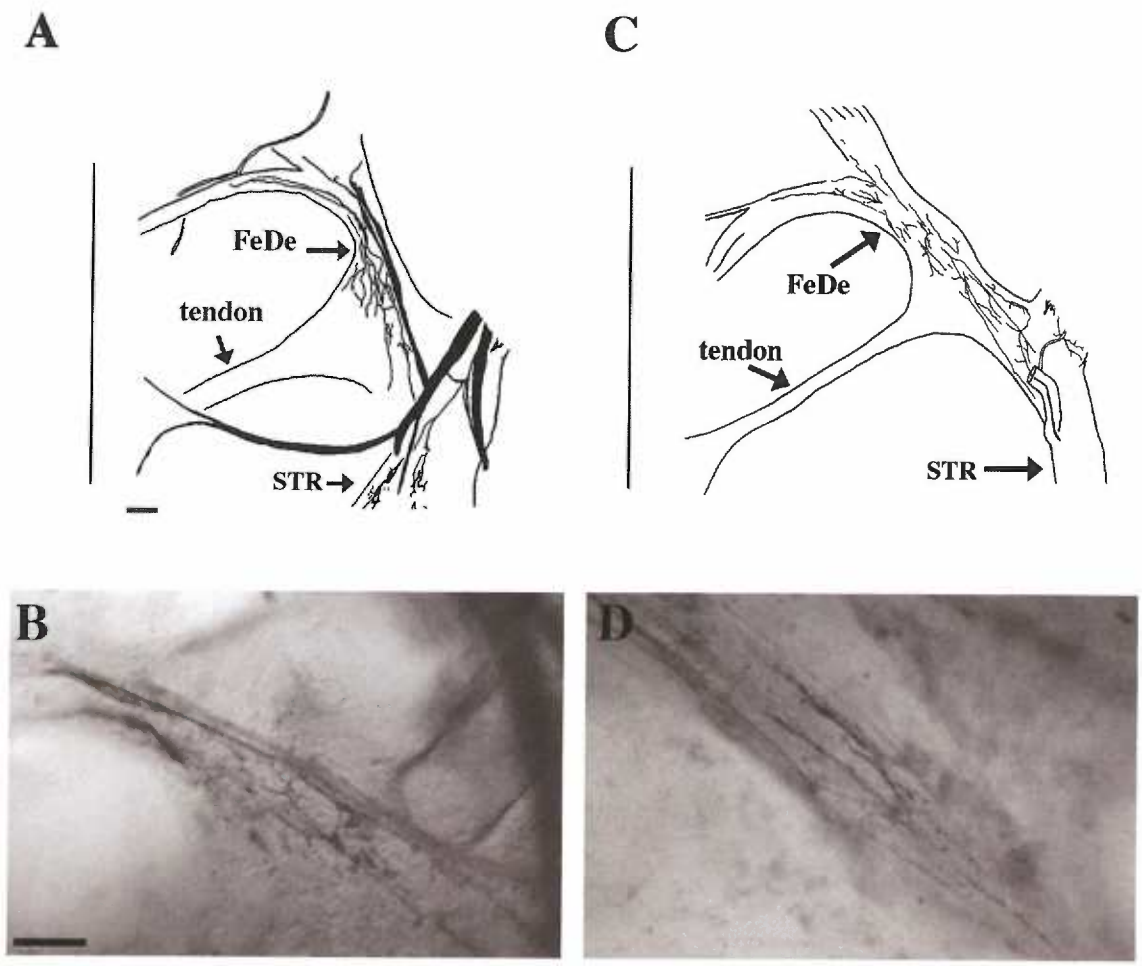

Figure 6. Anti-MFas II labeling associated with anlagen of the adult FeDe muscles in P1 pupae. (A, C) Camera lucida drawings of the muscle anlagen and its associated tendon in P1 pupae showing anti-GPI-MFas II labeling (A) and anti-TM-MFas II labeling (C). Bar, 100 μm. (B,D) Photographs of whole-mount FeDe muscle anlagen. Bar, 100 μm. Immunostaining with anti-GPI-MFas II (B) reveals thick proximal branches tapering to fine tips (arrow), while immunostaining with anti-TM-MFas II (D) labels only thin processes on the FeDe anlagen that have varicosities (arrow) associated with them.

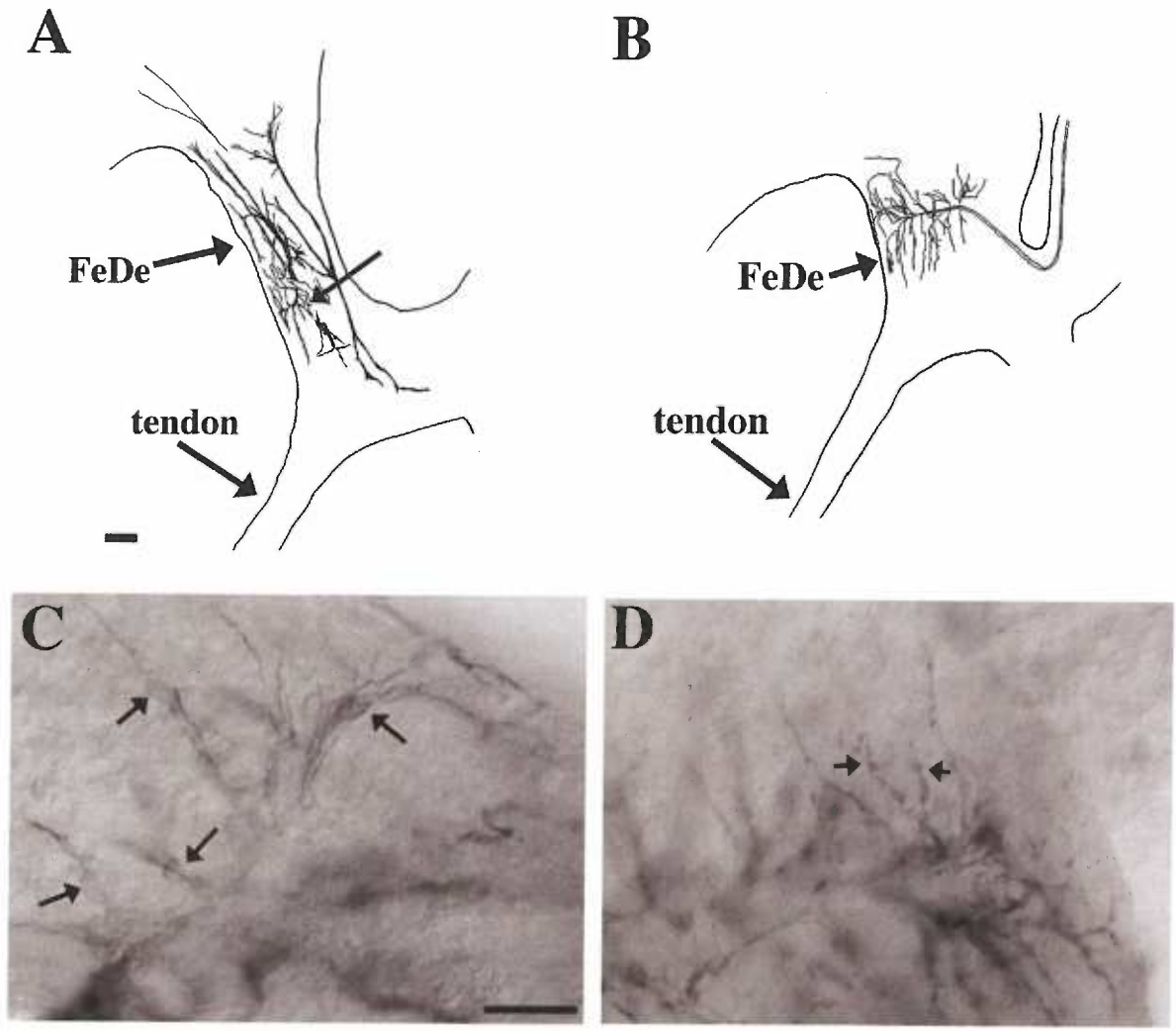

Figure 7. Anti-MFas II labeling associated with developing adult FeDe muscles in P3 pupae. (A, B) Camera lucida drawings showing anti-GPI-MFas II labeling of thick and thin processes (A) and anti-TM-MFas II labeling of only thin processes (B). Arrow in (A) indicates central area of muscle anlagen containing new growth. Bar, 100 μm. (C,D) Photographs of whole-mount FeDe muscle fibers. Bar, 100 μm. (C) Anti-GPI-MFas II labeling reveals profiles resembling glial cell bodies along the processes (arrows). (D) Anti-TM-MFas II labels thin processes that contain varicosities along their length (arrows).

across the muscle. By stage P7, the adult FeDe muscle had separated into thin, unstriated muscle fibers. Nerve branches extended over the entire surface of the muscle rather than being restricted to the central region of the muscle as seen at earlier stages. Anti-GPI-MFas II labeling was intense all along the length of the processes (Fig. 8A). In contrast, double-labeling and confocal microscopy revealed that anti-synaptotagmin labeling was becoming restricted to the distal portions of the processes (Fig. 9C). Similarly, anti-TM-MFas II labeling became restricted to the higher order neurites in the distal regions of the FeDe muscle and many of these appeared to contain differentiating boutons (arrow, Fig. 8B).

Differentiation of adult motor terminal morphology

Between stages P9 and P10, striations became apparent in the developing adult FeDe muscle fibers. Anti-GPI-MFas II continued to label the entire extent of the nerve, including transverse branches and tiny processes that appeared to be associated with the developing endplates (arrow, Fig. 8C). Anti-TM-MFas II labeling was restricted to fine UM-like processes, reminiscent of the staining pattern observed during larval stages. A single fine axon was labeled in the main nerve (arrow, Fig. 8D), but few varicosities could be detected on the muscle fibers (not shown). Central neurons, including a posterodorsal soma in the correct position to be the FeDe MN, continued to label with anti-TM-MFas II until P12 (not shown).

Between P15 and the adult stage, anti-GPI-MFas II labeling became progressively fainter in the motor branches and was particularly difficult to detect in the higher order branches when visualized with DAB (not shown). However, double-labeling and confocal microscopy revealed that anti-GPI-MFas II labeling extended to the higher order

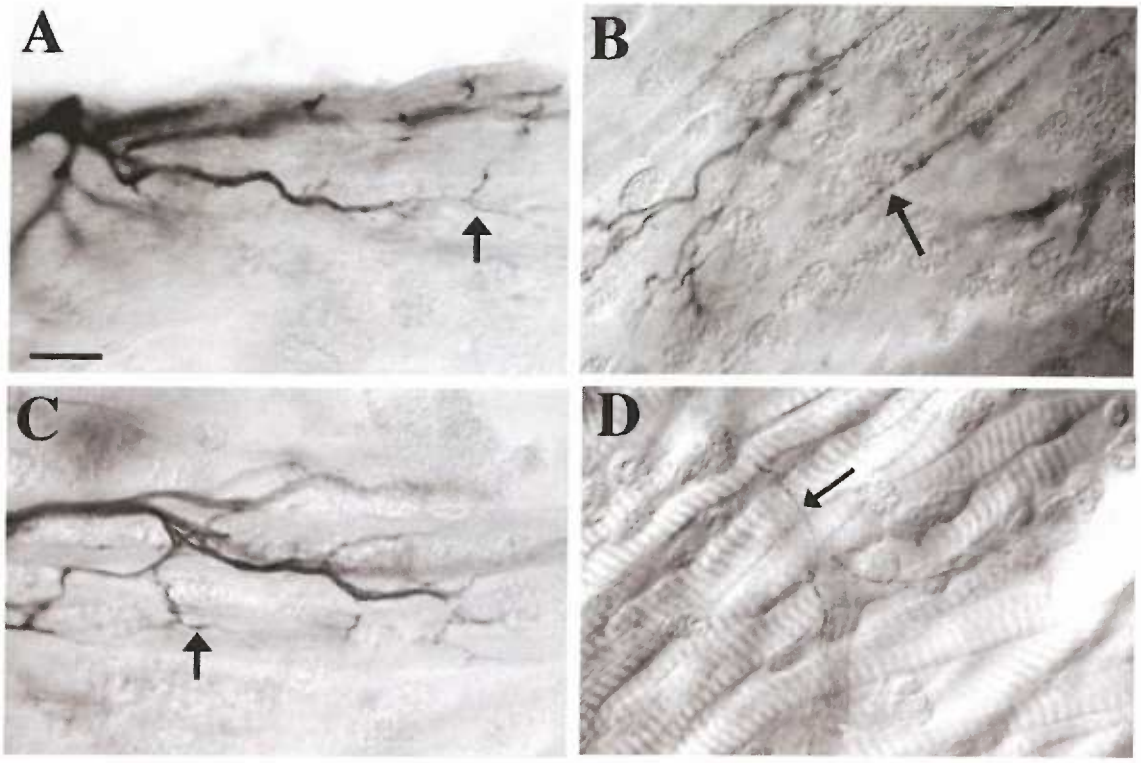

Figure 8. Photographs of developing adult FeDe muscles in P7 and P9/P10 pupae. Bar, 50 μm. (A) At P7, anti-GPI-MFas II continues to label the entire extent of the nerve, including the fine tertiary processes (arrow). (B) Anti-TM-MFas II labeling is still associated with secondary and tertiary nerve processes, as well as primary branches in the central region of the muscle. Terminal varicosities are also labeled (arrow). (C) At stage P9/10, anti-GPI-MFas II labeling continues to label the entire extent of the nerve, including the tiny distal processes (arrow). (D) Anti-TM-MFas II labeling is still detectable at P10, but is restricted to thin UM-like axons in the nerve (arrow) and few scattered boutons (not shown).

branches and was also associated with the terminal varicosities in the adult (arrows, Fig. 9D). As in the larval stage, anti-GPI-MFas II-labeled regions surrounding the anti-synaptotagmin labeled boutons. Rather than forming a rosette-like pattern, however, the boutons were arranged in linear patterns characteristic of the adult neuromuscular junction (Consoulas et al., 1996). Labeling with anti-TM-MFas II was no longer detectable in any pattern that resembled synaptic varicoscities or UM-like processes (not shown).

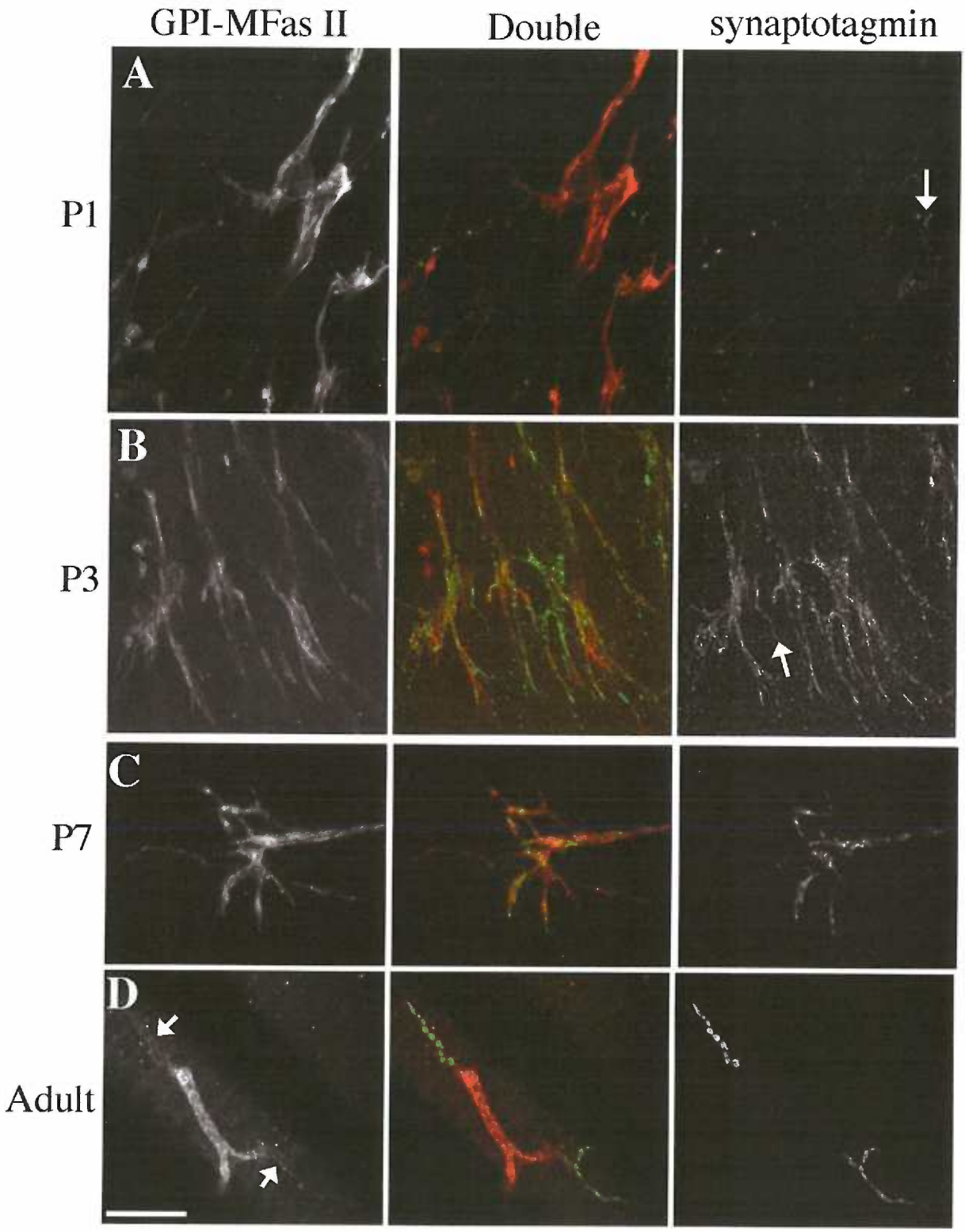

Figure 9

DISCUSSION

Labeling with isoform-specific anti-MFas II antisera has provided a new method for labeling motor nerves in *Manduca*, and has allowed us to identify distinct components associated with the peripheral nerves and neuromuscular junction. In addition, we have been able to monitor the remodeling of motor terminals during metamorphosis using an immunocytochemical approach, rather than dye-filling methods that are often lengthy and capricious. Thus, our results support and extend previous studies in which biocytin was used to monitor the changes taking place in leg motor terminals during metamorphosis (Consoulas et al., 1996). The anti-GPI-MFas II antisera allowed us to visualize motor nerves and branches at all stages, and appeared to be associated with the glial cells ensheathing the nerves. In addition, anti-GPI-MFas II antisera labeled regions associated with type I synaptic boutons, indicating that GPI-MFas II is localized to the endplate region, possibly to the processes of endplate glial cells. In contrast, the anti-TM-MFas II antisera provided a more dynamic view of motor-terminal remodeling, labeling motor axons only during prepupal and early pupal stages as they underwent retraction and new growth. Anti-TM-MFas II also labeled a second class of synaptic terminal on larval leg muscles, the type II terminals of the UM neurons, allowing us to identify the putative modulatory innervation of the larval leg muscle.

Isoform specific anti-Fas II antisera label different components of the neuromuscular junction

In the larval stage, anti-GPI-MFas II immunostaining revealed the branching pattern of the entire leg nerve as well as the rosette-like array of synaptic boutons associated with type I terminals. Thus, the pattern of labeling was similar to that

obtained by staining the entire leg nerve with biocytin, except that the anti-GPI-MFas II labeling formed an outline around the boutons, where it was less intense than along the nerve branches. Cell bodies of glial cells ensheathing the motor branches were clearly labeled with anti-GPI-MFas II (see Fig. 3B) while cell bodies of central neurons were not labeled, leading us to conclude that this labeling was associated with glia. Studies on embryonic populations of neurons and glia in *Manduca* have also shown that glial cells express GPI-MFas II, while neurons express TM-MFas II (Wright and Copenhaver, unpublished data; Wright and Copenhaver, 2000). In addition, other *Manduca* Fas II antisera have been reported to label the glial sheath surrounding motor axons during postembryonic life (Hegstrom, 1996b).

In contrast to the labeling along the leg nerves, the anti-GPI-MFas II-labeled regions outlining the synaptic boutons did not uniformly label the endplate glia, suggesting that those regions might represent a localization of GPI-MFas II to glial processes. Ultrastructural studies of the neuromuscular junction of flight muscle in *Manduca* indicate that terminal glial cells cap the synaptic boutons and that finger-like glial processes interdigitate between sites of direct contact between nerve terminals and muscle processes (Rheuben, 1992). Endplate glial cells labeled immunohistochemically with an anti-cGMP antibody have also been described on the larval abdominal muscle DEO1 (Hegstrom, 1996b). Here, we used an anti-neuroglian antibody (Chen et al., 1997; Nardi, 1994) to reveal the endplate glial cells, demonstrating that the anti-GPI-MFas II labeling associated with the boutons was encompassed by the broader anti-neuroglian labeling around the terminals. Localized expression of GPI-MFas II on glial processes, however, could account for some of the labeling, perhaps providing for homophilic

adhesive interactions between the glial processes and TM-MFas II expressing neuronal processes. Postsynaptic localization of GPI-MFas II in muscle processes could also account for the staining pattern we observed. Hence, GPI-MFas II may also be expressed by muscle, becoming localized to postsynaptic sites as synapses mature, much like TM-Fas II becomes localized postsynaptically in *Drosophila* (Schuster, 1996a). Although we did not rule out presynaptic localization of GPI-MFas II within the nerve terminals, we believe neuronal expression of GPI-MFas II is unlikely. Transient neuronal expression of GPI-MFas II was detected in the enteric neurons (EP cells) prior to their migration in embryonic *Manduca*, but once migration had begun the neurons expressed only TM-MFas II and the glial cells expressed only GPI-MFas II (Wright and Copenhaver, 2000). Moreover, cell culture studies have indicated that neurons dissociated from larval or pupal thoracic ganglia express TM-MFas II but not GPI-MFas II (unpublished observations). Without an ultrastructural analysis, however, the cellular elements expressing GPI-MFas II cannot be determined. Hence, the possibility remains that the labeling around the boutons is contributed by some combination of nerve terminals, glial processes, and muscle processes, perhaps providing for homophilic adhesive interactions between them.

During larval stages, anti-TM-MFas II labeled thin processes that resembled type II terminals, characteristic of innervation by the UM neurons (see Fig. 2E). The FeDe muscle bundles, like all leg muscles, are also innervated by an UM neuron that has its soma located on the posterior midline of the ganglion. The terminals of the UM neurons are simple and lack the rosette-like array characteristic of the larval leg motoneurons (Consoulas et al., 1999). We did not trace individual UM axons from the CNS to the

FeDe muscles, but UM somata and processes in the CNS were among the few neuronal components labeled with anti-TM-MFas II during the larval stage, supporting our conclusion that the thin processes associated with the larval FeDe muscles belonged to the UM neurons.

In the adult, labeling with anti-GPI-MFas II did not reveal areas outlining synaptic boutons when immunoreactivity was visualized with DAB reaction product, though labeling was still detectable on the major nerve branches. Confocal microscopy, however, did reveal labeling of the higher order branches and areas associated with boutons (see Fig. 9). Whether the level of expression of the MFas II isoforms is lower in the adult or whether other differences reduce the intensity of the labeling is unclear. Glial cells are also known to be present at the neuromuscular junction of flight muscle in adult *Manduca* (Rheuben, 1985). Thus, as in the larva, the anti-GPI-MFas II labeling surrounding the synaptic boutons in the adult could be associated with glial processes and/or post-synaptic components of the neuromuscular junction.

We were unable to detect any anti-TM-MFas II labeling of axonal processes on the FeDe muscle fibers in the adult stage. Why the UM processes labeled in the larval stages but not in the adult stage is unclear. During larval stages, muscle growth occurs during each instar, and expression of the TM-MFas II isoform by the UM neurons might be associated with continual longitudinal growth of their processes. In the adult stage, there is no further growth of the muscles, and the lack of expression of the TM-MFas II isoform may reflect the lack of further longitudinal growth of the UM processes. In like manner, the expression of TM-MFas II by embryonic neurons is largely confined to periods of active outgrowth (Wright and Copenhaver, 2001).

The expression patterns of both Fas II isoforms in *Manduca* point to differences between the organization of the neuromuscular junction in *Manduca* and *Drosophila*. In *Drosophila* larvae, TM-Fas II is localized pre- and post-synaptically at type I synapses (Davis et al., 1997; Schuster, 1996a; Schuster et al., 1996b) and is also localized at least pre-synaptically at type II boutons (Zito et al., 1997). In larval *Manduca*, TM-MFas II is expressed throughout the axonal processes and boutons of the type II terminals, but localized expression at type I boutons was difficult to detect. Moreover, the GPI-MFas II isoform is associated with both type I and type II terminals in larval *Manduca*. Another notable difference between the neuromuscular junctions of the two insects is that motor terminals in *Manduca* are capped by endplate glial cells. Presynaptic terminals in *Drosophila* are not covered by glia, but rather are invested in a subsynaptic reticulum that consists of layers of convoluted muscle processes (Atwood et al., 1993; Auld et al., 1995). In *Manduca*, the subsynaptic reticulum includes glial processes that separate nerve-muscle contacts from each other (Rheuben, 1992). The GPI-MFas II labeling at the *Manduca* neuromuscular junction, therefore, might reflect expression localized to glial processes that separate sites of nerve-muscle contact.

In this regard, the organization of the neuromuscular junction in *Manduca* may be more similar to the organization found in vertebrates. In vertebrates, glial cells (terminal Schwann cells) also cap the synaptic terminals at the neuromuscular junction and express neural cell adhesion molecule (NCAM), the vertebrate ortholog of Fas II (Grenningloh et al., 1991). Both TM isoforms and GPI-linked isoforms of NCAM exist, but, in contrast to the situation in *Manduca*, only the TM-NCAM isoform is associated with Schwann cells as well as with the nerve and muscle. Although the neuromuscular

junctions of mice that lack NCAM seem to develop normally, the absence of NCAM is associated with several defects in the development of normal synaptic structure and physiology (Bixby and Reichardt, 1987; Moscoso et al., 1998; Rafuse et al., 2000). In addition, experiments with function-blocking antibodies suggest that NCAM may play a role in the determination of synaptic areas and Schwann cell-axon interactions during regeneration (Rieger et al., 1988). Whether either isoform of MFas II plays a similar role in *Manduca* awaits additional studies.

MFas II expression during motor-terminal retraction

Labeling with anti-GPI-MFas II antisera during the prepupal stages revealed features similar to those observed with biocytin staining (Consoulas et al., 1996), showing that motor terminals began to retract and synaptic boutons appeared to coalesce into larger varicoscities. By W+3, the rosette-like endplates were gone, but remnants of degenerating endplates labeled with anti-synaptotagmin appeared to be ensheathed by GPI-MFas II positive processes. This result is similar to that reported by Hegstrom and Truman (1996) and supports their suggestion that contents of the synaptic boutons are actively retracted into tertiary branches of the axonal arbor, which are surrounded by the glial sheath. However, we also observed other varicosities that ranged in size and appeared to be devoid of GPI-MFas II labeling (see Fig. 3E, F). Whether those varicosities lacked an association with glial cells or were associated with isolated glial cells that had stopped expressing GPI-MFas II could not be determined. In either case, the presence of apparently isolated varicosities suggests that loss of endplates might involve more than a coalescence and retraction into the glial sheath.

Because expression of TM-Fas II is associated with outgrowth of motor axons during embryonic development of *Drosophila*, we anticipated that TM-MFas II might be associated with motor-terminal growth during pupal stages. However, we were surprised to observe an increase in anti-TM-MFas II-labeling during early phases of motor-terminal retraction, between W+1 and W+2 (see Figs. 4B-C). While this apparent upregulation might indicate a role for TM-MFas II during retraction, it could also be associated with an early growth phase that occurs prior to pupation (see below). Thus, the anti-TM-MFas II-labeled tufts that we observed by W+3 could represent motor processes that began to grow while others were still retracting. Concomitant retraction and growth has also been reported to occur during metamorphosis of silkmoth flight muscle, such that larval terminals appeared to be retracting while new processes were sprouting from other branches (Stocker and Nüesch, 1975).

MFas II expression during motor-terminal growth and differentiation of endplates

Our immunocytochemical labeling with both anti-MFas II antisera indicated that the terminals associated with the FeDe muscle remnant expanded by pupation, but then underwent additional pruning by P4 (compare Figs 6C and 7B). Similar results obtained with biocytin labeling were reported for the more distal leg muscles (Consoulas et al., 1996). Labeling with anti-synaptotagmin revealed only a few varicosities associated with thin processes on the muscle remnant, while labeling with both anti-MFas II antisera revealed numerous processes associated with the remnant on P1 (see Figs. 6, 8). Thus, many of the processes may not have contained an accumulation of synaptic vesicles or their precursors during this period, perhaps associated with a transient downregulation of synaptotagmin. Anti-GPI-MFas II labeled thick as well as thin processes, once again

suggesting that the labeling was associated with glial cells ensheathing the motor branches. In contrast, anti-TM-MFas II labeled only thin processes, which were fewer than those labeled with anti-GPI-MFas II. Thus, some of the anti-GPI-MFas II-labeled processes might have been the remnants of the glial sheath left after axon branches degenerated. Because both the TM-MFas II and GPI-MFas II antisera were generated in the same host species, we were unable to double-label preparations to evaluate this interpretation. Hegstrom and Truman (1996) have also reported that apparently empty glial sheaths remain in place after degeneration of axon branches.

By P3-P4, a new growth phase was apparent. A dramatic increase in anti-synaptotagmin immunoreactivity occurred, such that both anti-TM-MFas II and anti-synaptotagmin labeled numerous thin processes that appeared to be sprouting from a thick primary branch centrally located on the muscle anlagen. Anti-GPI-MFas II labeled thicker processes with similar orientations, suggesting that glial cells and processes were closely aligned with most of the neurites. Double-labeling with anti-GPI-MFas II and anti-synaptotagmin supported this conclusion (Fig. 9B). Whether some neuronal processes expressed GPI-MFas II in addition to the peripheral glial cells could not be determined with our methods, but double-labeling indicated that some anti-synaptotagmin immunoreactive neurites were not associated with anti-GPI-MFas II labeling (arrow, Fig. 9B). The small diameter of those anti-synaptotagmin labeled processes suggested that they might be filopodia extending from growing neurites.

Previous work has demonstrated a close association between glial cells and outgrowing motor axons. Ultrastructural studies on the developing innervation of silkmoth flight muscle indicated that glial cells and their processes were intimately

associated with outgrowing axons at all stages (Stocker and Nüesch, 1975). During embryonic development of *Manduca*, glial cells appear to follow the motor axons in some nerves, but prefigure axonal pathways for others (Carr and Taghert, 1988; Wright and Copenhaver, 2001). During development of the vertebrate neuromuscular junction, glia follow the axons during axon outgrowth, but after axotomy, the glial cells provide a channel for reinnervating axons (reviewed by Sanes and Lichtman, 1999). Because we observed apparently empty glial sheaths at one stage and "unsheathed" neurites at another, our results suggest that both scenarios could occur during the metamorphic re-growth of motor terminals. Thus, empty glial sheaths could provide channels for initial axonal outgrowth, but glial cells and their processes might subsequently follow the outgrowing neurites.

While extensive longitudinal growth of the motor processes occurred between P5 and P7, such that branches reached the distal regions of the developing muscle, labeling with anti-TM-MFas II and anti-synaptotagmin became progressively restricted to higher order branches. Localization of synaptotagmin to terminal varicoscities has been reported previously in *Drosophila* (Littleton et al., 1993) and in *Manduca* (Consoulas et al., 1996; Consoulas and Levine, 1998). Similarly, during embryonic development in *Drosophila*, TM-Fas II is initially expressed on all motor axons and their growth cones (Van Vactor et al., 1993), but subsequently becomes localized to the synapse, both pre- and postsynaptically (Davis et al., 1997; Schuster et al., 1996b). Instead of becoming localized to the synapse in *Manduca*, however, anti-TM-MFas II labeling became undetectable. Thus, by P10, when the overall adult form of the muscle was attained and muscle fibers became striated, anti-synaptotagmin labeling was restricted to terminal

branches and varicosities, but labeling with anti-TM-MFas II was difficult to detect in the developing motor terminals. The decrease in anti-TM-MFas II immunoreactivity in motor terminals by P10 is likely to reflect the level of maturity obtained by the growing motor processes at that stage. Thus, progressive localization of synaptic vesicles or their precursors and localization or downregulation of TM-MFas II appeared to be coincident with a transition from longitudinal growth to the differentiation of adult junctions.

In contrast to the reduction in anti-TM-MFas II-labeling observed during the first half of the pupal stage, anti-GPI-MFas II labeled the growing processes intensely until a few days prior to adult eclosion. An apparent downregulation of GPI-MFas II occurred a few days prior to eclosion, such that labeling became progressively less intense and could not be detected in association with terminal boutons without the use of confocal microscopy. This apparent downregulation coincided with the final maturation of the adult terminals and the restriction of synaptotagmin to terminal varicosities. Ultrastructural studies of the silkmoth neuromuscular junction (Stocker and Nüesch, 1975) also indicated that terminal glial cells shrank during the last few days prior to eclosion. Hence, GPI-MFas II localized to processes of terminal glial cells might label less intensely in mature preparations as a result of similar structural changes.

Conclusion

In summary, the use of isoform-specific antisera has allowed us to examine nerve terminal remodeling during metamorphosis by focusing on distinct cellular elements at the neuromuscular junction. Our results demonstrate that TM-MFas II, a molecule expressed on the growing processes of motoneurons during embryonic development, is re-expressed on the processes of leg motoneurons during metamorphosis, perhaps to

mediate re-growth of motor axons across the developing adult muscle. In contrast, GPI-MFas II appears to be expressed by glial cells that ensheath the peripheral nerves at all stages of development and is also localized to mature synaptic endplates, possibly contributing to the structural and/or functional integrity of nerve terminals.

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CHAPTER 2

Remodeling of an Identified Motoneuron During Metamorphosis: Central and Peripheral Actions of Ecdysteroids During Regression of Dendrites and Motor Terminals

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SUMMARY

During metamorphosis of the moth *Manduca sexta*, an identified leg motoneuron, the femoral depressor motor neuron (FeDe MN), undergoes reorganization of its central and peripheral processes. This remodeling is under the control of two insect hormones: the ecdysteroids and juvenile hormone (JH). Here, we asked whether peripheral or central actions of the ecdysteroids influenced specific regressive aspects of MN remodeling. We used stable hormonal mimics to manipulate the hormonal environment of either the FeDe muscle or the FeDe MN soma. Our results demonstrate that motor-terminal retraction and dendritic regression can be experimentally uncoupled, indicating that central actions of ecdysteroids trigger dendritic regression whereas peripheral actions trigger terminal retraction. Our results further demonstrate that discrete aspects of motor-terminal retraction can also be experimentally uncoupled, suggesting that they also are regulated differently.

INTRODUCTION

During metamorphosis of holometabolous insects, such as moths and flies, neuromuscular systems undergo a hormonally-mediated transformation. In extreme cases, larval muscles undergo complete histolysis and new adult muscles develop from muscle precursor cells. Many of the motoneurons (MNs) that innervated these degenerated larval muscles are remodeled to innervate the new adult muscles. In the central nervous system (CNS), the larval dendrites regress and are replaced by a new adult dendritic arbor. In the periphery, larval motor terminals are lost as the larval muscle degenerates, and new adult terminals differentiate as the new adult muscle develops. Both the MNs and muscles are targets of hormone action, so hormones may influence MN remodeling via central and peripheral actions. The goal of the current study was to determine the influence of peripheral hormone actions on the central and peripheral remodeling of an identified MN.

Insect metamorphosis is controlled hormonally, primarily by the ecdysteroids (principally the steroid hormone 20-hydroxyecdysone, 20E; reviewed by Gilbert et al., 1996; Riddiford, 1994). Reprogramming of the larval tissues to a pupal fate occurs during the final (fifth) larval stage when a small surge in ecdysteroids occurs for the first time in the absence of another hormone, juvenile hormone (JH). This "commitment pulse" of ecdysteroids is followed by a second, larger surge of ecdysteroids called the "prepupal peak", which occurs in the presence of JH. The prepupal peak of ecdysteroids initiates the breakdown of larval-specific tissues, the formation of a pupal cuticle, and the molt to the pupal stage. A final surge in the ecdysteroid titer called the pupal peak occurs

during the pupal stage in the absence of JH, and this pupal ecdysteroid titer directs development of the adult.

The remodeling of the neuromuscular system that occurs during metamorphosis is also controlled by the ecdysteroids. Death of larval MNs and/or regression of MN dendrites require both the commitment pulse and the prepupal peak of ecdysteroids (Weeks and Truman, 1985; Weeks and Truman, 1986). Growth of new dendrites of respecified MNs requires the pupal peak of ecdysteroids (Truman and Reiss, 1988; Weeks and Ernst-Utzschneider, 1989). Several experiments suggest that dendritic changes are triggered by central ecdysteroid action rather than by peripheral effects on cellular targets or sensory inputs (Jacobs, 1990; Kent and Levine, 1993; Weeks and Truman, 1985). Results from *in vitro* studies support the idea that ecdysteroids act directly on MNs, at least to enhance process complexity (Matheson and Levine, 1999; Prugh, 1992; Witten and Levine, 1991). This is in contrast to steroid-sensitive neuromuscular systems in vertebrates where steroid hormones increase dendritic complexity of MNs indirectly by acting on their target muscles (Rand and Breedlove, 1995). Soma size, however, appears to be influenced by direct hormone action on the MNs (Watson et al., 2001).

Cell-cell interactions may also affect specific aspects of neuronal remodeling that take place during metamorphosis. Although interactions with target muscle appear to be neither necessary nor sufficient to trigger dendritic regression (Weeks, 1987; Weeks and Truman, 1985), signals from target muscle might modulate dendritic changes (Kent and Levine, 1993). Moreover, interactions with target muscle appear to influence specific aspects of the peripheral changes that occur in the MN axonal branches and terminals

(Hegstrom, 1996b; Truman and Reiss, 1995). In one study, the topical application of a JH mimic (JHM) to body wall muscles during the pupal stage led to the growth of larval-like muscles in the adult (Truman and Reiss, 1995). Although larval endplates were lost, the larval-like muscles were associated with axon branches that should have been lost, which suggested that local interactions with target muscle could influence axonal pruning (Truman and Reiss, 1995). Whether the loss of larval endplates could be prevented by preventing the initial histolysis of the larval muscle was not examined. In another study, however, the application of the non-steroidal ecdysteroid mimic RH5992 to larval muscles resulted in local histolysis of the larval muscle and loss of synaptic endplates (Hegstrom, 1996b), suggesting that signals from the degenerating muscle might be sufficient to induce the loss of synaptic endplates. Whether central ecdysteroid action was necessary to render the MN competent to respond to the peripheral signals was not determined.

To address unresolved questions regarding the influence of peripheral tissues on motor-terminal retraction, we have focused on an identified leg MN, the metathoracic femoral depressor MN (FeDe MN), which has been well-characterized in terms of its dendritic remodeling (Kent and Levine, 1988a; Kent and Levine, 1993), and its motor-terminal remodeling (Knittel et al., 2001). Here, we have used hormonal manipulations to examine the central and peripheral role of the ecdysteroids in the process of motor-terminal retraction. Our studies differ from previous studies in several ways. First, we have been able to prevent the degeneration of a larval muscle through the local application of JHM prior to the commitment pulse so that the FeDe MN is exposed to ecdysteroids centrally without concurrent degeneration of its larval target muscle.

Second, we have developed a new type of heterochronic mosaic by injecting JHM into the larval metathoracic ganglion to prevent the pupal commitment of the neurons without altering the hormonal environment in the periphery. Finally, we have examined the dendrites and motor terminals of the same FeDe MN in our hormonally-manipulated animals in order to assess the effect of our hormone treatments on both the CNS and periphery.

Our results indicate that interactions with target muscle do not modulate ecdysteroid-induced dendritic regression but do exert an influence on motor-terminal retraction. Ecdysteroid action on the MN somata in the CNS is not sufficient or necessary to trigger motor-terminal retraction, but the MN must be rendered competent by the commitment pulse of ecdysteroids to respond completely to peripheral cues. Preliminary descriptions of these results have been reported in abstract form (Kent and Bollermann, 1995; Knittel et al., 2001; Wilson and Kent, 1998).

MATERIALS AND METHODS

Animals

Manduca sexta (Lepidoptera: Sphingidae) were obtained from a laboratory culture reared on an artificial diet (Bell and Joachim, 1976) and a long-day photoperiod regimen (17:7 h light/dark) at 26 ^{o}C and approximately 60% relative humidity. Developmental stages were monitored by both chronological and morphological criteria (Nijhout and Williams, 1974; Reinecke et al., 1980; Tolbert et al., 1983; Truman and Riddiford, 1974; Truman et al., 1980). For the prepupal stages, W0 signifies the first day of the prepupal stage or "wandering," and W1, W2, etc., refer to the ensuing prepupal days. W0 stage larvae were identified by the exposure of the dorsal vessel. P0 designates the day of pupation. Animals maintained under our rearing conditions typically pupated on day 4 or 5 after the onset of wandering. Prior to surgical or experimental manipulation, animals were anesthetized either by immersion in ice water or exposure to CO_2.

Hormonal manipulations

To eliminate the source of ecdysteroids, larvae were anesthetized at stage W0 and ligated with silk suture through the middle of the mesothoracic segment. The head and prothoracic segment were excised and discarded. Four to six days after ligation, the metathoracic FeDe MN was intracellularly labeled using hexamine-cobalt injections as described below, and/or the metathoracic legs were dissected and fixed for immunocytochemistry.

To distinguish between peripheral and central influences of ecdysteroids on motor-terminal retraction, we created three different types of heterochronic mosaic insects in which peripheral tissues and the metathoracic ganglion were at different developmental

stages. In the first type of heterochronic mosaic, one metathoracic leg was developmentally arrested at the larval stage while the remainder of the animal underwent pupal development. In an earlier set of experiments, JHM (the juvenile hormone mimic S-methoprene; gift of Sandoz, Inc.) was mixed with melted dental wax (Periphery Wax, Surgident) at a concentration of 1-2 μg/ml and applied externally to one or both larval legs on the first day of the fifth larval instar, according to methods originally developed by Levine et al., (1986). While this treatment preserved larval legs including the leg sensilla and the associated sensory neurons, the leg muscles often underwent histolysis. In the current study, larval leg muscles were preserved by injection of the JHM in mineral oil (1μg/ml) into one or all segments of the left larval leg on the first day of the fifth instar. Mineral oil alone was injected into the contralateral leg as a control.

In a second type of heterochronic mosaic, larvae were developmentally arrested at stage W0, while one metathoracic leg was induced to undergo pupal development. Larvae were deprived of their prothoracic glands by ligation through the mesothoracic segment at stage W0. One day later, the ecdysteroid mimic RH5992 (10 mg/ml in DMSO, mixed 1:1 with mineral oil; gift of Rohm and Haas, Inc.) was injected into the larval femur and/or coxa to trigger degeneration within that leg. RH5992 belongs to a class of compounds, dibenzoyl hydrazines, that have been shown to act as ecdysteroid agonists by interactions with the ecdysteroid receptor (Wing, 1988; Wing et al., 1988). The effects of RH5992 in tissue persist much longer than 20E (Retnakaran et al., 1995), and RH5992 has been used to induce premature muscle histolysis in larval abdominal muscle in *Manduca* (Hegstrom, 1996b).

In the final type of heterochronic mosaic, one larval ganglion was arrested in the larval stage while the remainder of the animal underwent pupal development. The metathoracic ganglion was injected with JHM (1μg/ml in mineral oil) on the first day of the fifth instar to prevent pupal commitment of the FeDe MN. Metathoracic ganglia were surgically exposed, immobilized on a platform, and pressure-injected with 0.1-0.3 μl of JHM or mineral oil using a PicoPump (WPI, Inc.). The incision was sutured closed and sealed with Vetbond (3M). Animals were dissected on the first day of pupation.

Fluoro-Gold labeling of the FeDe muscle

To visualize the FeDe muscle in hormonally-manipulated insects, we injected a 5% aqueous solution of Fluoro-Gold (Fluorochrome, Inc.) into the larval leg on the first day of the fifth instar (Schmued and Fallon, 1986). Four to seven days after the injection, legs were dissected to reveal the FeDe muscle and fixed overnight in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The dissected preparations were rinsed in 0.1 M phosphate buffer (pH 7.4), dehydrated in ethanol, cleared in methyl salicylate, and examined with a compound microscope with a blue (DAPI) epifluorescence filter set.

Intracellular labeling of MNs

To visualize the dendrites of the FeDe MN in the CNS, we used standard intracellular dye-injection methods (Kent and Levine, 1993). The cell body of the FeDe MN was retrogradely labeled with a non-toxic, persistent fluorescent dye (DiI, 2.5 mg/ml ethanol; Rhodamine Dextran Lysine 3000 MW or Alexa 488 Dextran Lysine 10,000 MW, 5% in 2% KCl Molecular Probes) during the first few days of the last larval instar by pressure-

injection into the region of the FeDe muscle (Kent and Levine, 1993; Knittel et al., 2001). At the time of dissection, the fluorescently-labeled MN was located using an Olympus BHJM compound microscope equipped with epifluorescence. The MN was then impaled with a glass microelectrode that had a tip resistance of 20-40 MΩ when filled with a solution of hexamine cobaltic chloride (100 mM in 200 mM KAc). Following a standard 20 minute injection period with 10-15 nA of positive current, the cobalt was precipitated as cobalt sulfide, and the ganglion was fixed in Carnoy's solution (Humason, 1979). The preparations were subsequently processed according to modifications of the Timm's silver intensification method (Bacon and Altman, 1977), dehydrated and cleared with methyl salicylate, and mounted in Canada Balsam (Fisher Scientific). The dendrites of the MN were drawn with the aid of a *camera lucida* attachment on a Zeiss upright compound microscope.

Biocytin labeling of axons and terminals

To reveal the entire extent of the peripheral branching of the FeDe MN processes, a biocytin protocol was used, as described previously (Consoulas et al., 1996; Knittel et al., 2001). Briefly, animals were dissected in *Manduca* saline (Weevers, 1966), and the metathoracic ganglion was isolated from the surrounding saline in a petroleum-jelly well filled with distilled water. The ganglion was then severed from the leg nerves in a hypotonic environment to allow the infusion of a biocytin solution (3%w/v in distilled water; Sigma). The preparations were stored at 7° C for 2-4 days, and were subsequently dissected and fixed overnight in freshly prepared fixative solution (4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4). Motor terminals were visualized by incubating with

Oregon Green Avidin (Molecular Probes) overnight at 4°C and viewed using a Zeiss upright compound microscope with a FITC epifluorescence filter set.

Immunocytochemistry

To visualize the motor terminals, we used a *Drosophila* anti-synaptotagmin antibody (Dsyt2; generously provided by J.T. Littleton and H.J. Bellen), as described previously (Knittel et al., 2001). Briefly, following dissection in a zero-calcium saline (modified from Jacobs, 1990; Miyazaki, 1980), legs were fixed overnight in 4% paraformaldehyde. Anti-synaptotagmin was used at 1:1000 in phosphate buffered saline + 0.2% Triton X-100 and 5% normal goat serum, and was processed for immunohistochemistry using a biotinylated goat-anti-rabbit secondary antibody, an elite ABC kit (Vector Labs), and DAB as the substrate.

Laser Scanning Confocal Microscopy

In some cases, tissue was labeled with anti-synaptotagmin and imaged with a confocal microscope. Tissue was processed for immunohistochemistry as described above and an Alexa Fluor-488 conjugated goat-anti-rabbit secondary antibody (Molecular Probes) was used. In some preparations, leg nerves were also labeled with an anti-*Manduca* Fasciclin II (MFasII) antibody (made in Guinea Pig) that recognized only the GPI-linked isoform of MFasII (Knittel et al., 2001; Wright and Copenhaver, 2000). An Alexa-Fluor-594 conjugated goat-anti-Guinea Pig secondary antibody (Molecular Probes) was used to visualize the anti-GPI-MFasII labeling. Images were obtained using a BioRad MRC 1024 ES laser scanning confocal microscope (CLSM) attached to a Nikon Eclipse TE300 inverted fluorescent microscope fitted with a krypton/argon laser

generating excitation lines at 488, 568 and/or 647 nm. Optical sections of 1-5 μm were recorded through the depth of whole-mount preparations.

RESULTS

The sequence of events that occurs during degeneration of the larval FeDe muscle and retraction of the FeDe motor terminals has been described previously (Knittel et al., 2001). In general, muscle degeneration and motor-terminal retraction occur simultaneously, coincident with the prepupal peak of the ecdysteroids (Fig. 1). Briefly, the larval FeDe muscle remains intact through day W1, and the FeDe motor terminals retain their larval rosette-like endplates, as revealed by anti-synaptotagmin immunoreactivity (Fig. 2A-C). Degeneration of the larval FeDe muscle and retraction of FeDe motor terminals proceeds over the subsequent 2-3 days of the prepupal stage. On W2 degenerative changes are apparent in the FeDe muscle, such that muscle fibers and endplates appear condensed (Fig. 2D). On W3, muscle fibers completely degenerate, leaving a medial mass that becomes the adult muscle anlagen. Rosette-like endplates are gone on W3, but scattered enlarged anti-synaptotagmin positive varicosities are associated with the degenerating muscle fibers (arrows, Fig. 2E). Similarly, on the first day of the pupal stage (P0), a few enlarged anti-synaptotagmin positive varicosities are associated with the muscle anlagen (arrows, Fig. 2F). The concomitance of muscle degeneration with motor-terminal retraction suggested that signals from the degenerating muscle might control or influence motor-terminal retraction. Thus, we created heterochronic insects by altering the local hormonal environment of either peripheral tissues or the CNS to determine the contributions of central and peripheral ecdysteroid actions to motor-terminal retraction.

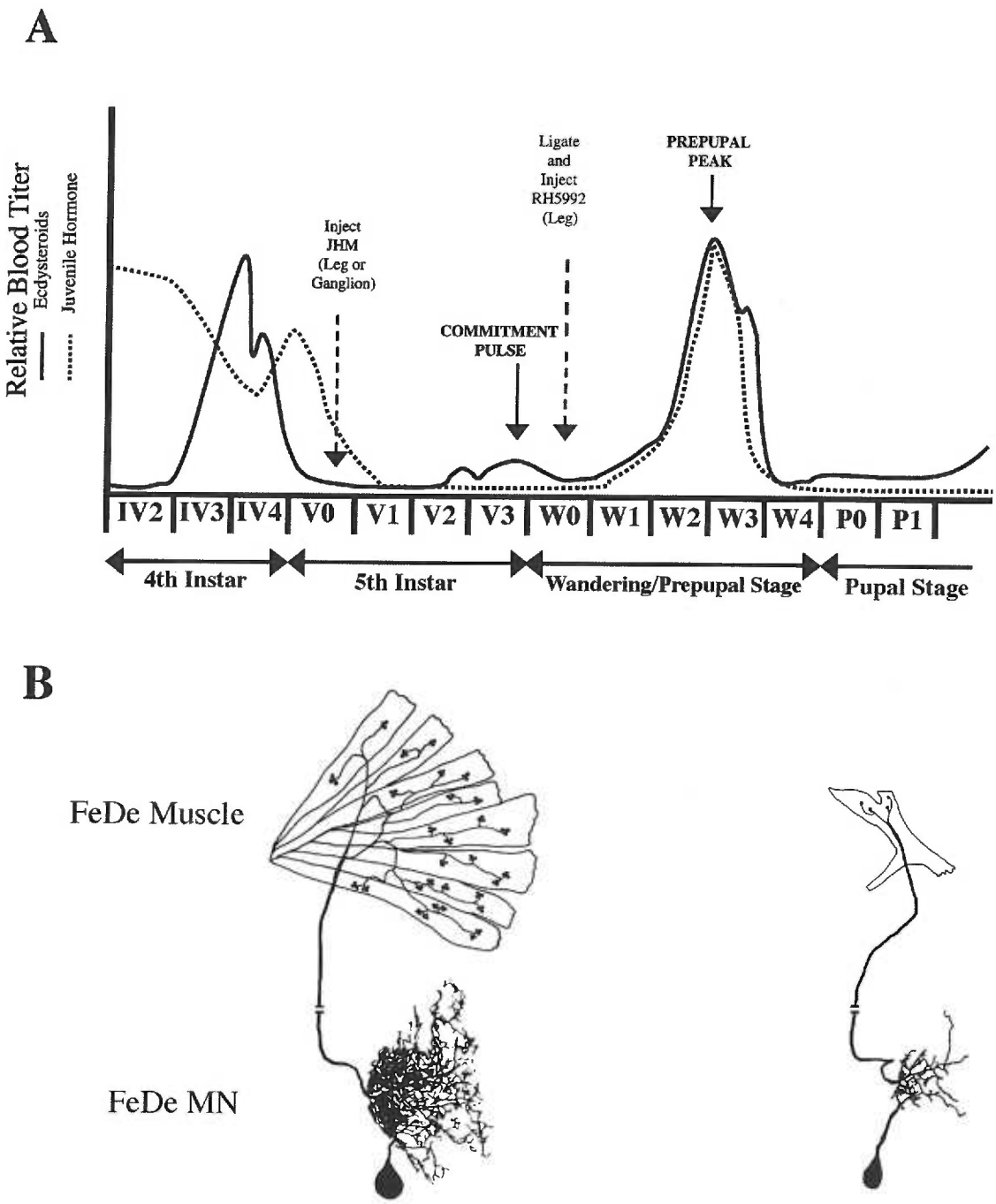

Figure 1. (A) Relative ecdysteroid and juvenile hormone (JH) titers during the larval-pupal transition. Times at which hormonal manipulations were performed to create heterochronic mosaics are indicated by arrows with dashed lines. (B) Morphology of the FeDe MN and FeDe muscle at larval stages and at P0. Titer data adapted from Riddiford, 1994. Dendritic morphology from Kent and Levine, 1993; muscle and motor-terminal morphology adapted from Knittel et al., 2001.

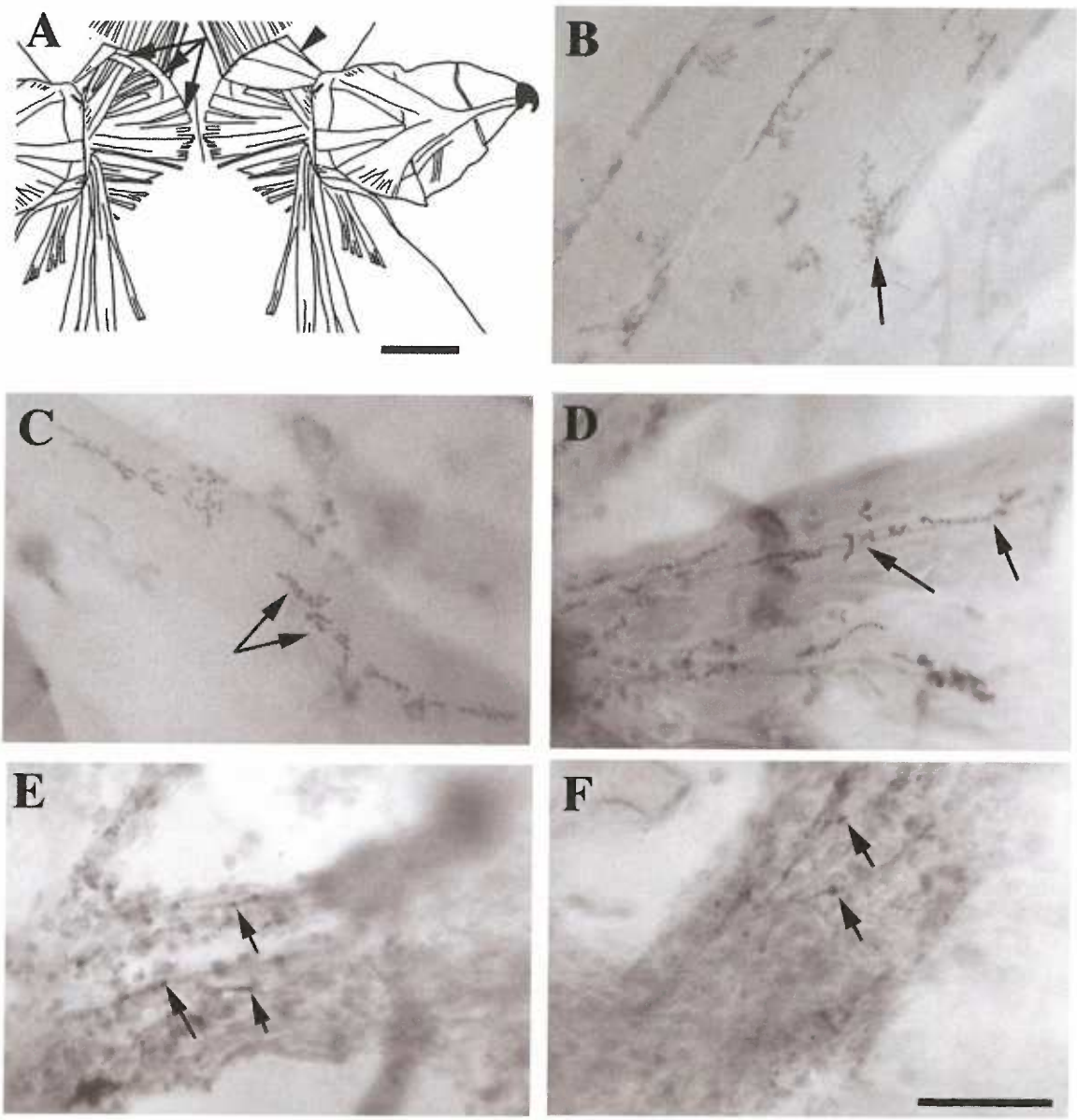

Figure 2. FeDe muscles in fifth-instar, prepupal, and newly pupated animals. (A) Illustrates the orientation of the FeDe muscles in this and subsequent figures. Arrows on the left indicate the three main FeDe muscle groups visible after dissection of overlying muscles. The arrowhead on the right indicates the antagonist FeLe muscle that is left intact in some of our preparations. (B-F) Anti-synaptotagmin labeling of motor terminals associated with the FeDe muscles in normal fifth-instar larvae (B) and at stages W1 (C), W2 (D), W3 (E), and P1 (F). Arrows indicate rosette-like endplates (B, C, D) and retracted varicosities (E, F). Scale bar = 1 mm in (A) and 100 μm in (F) [same as magnification in (B-E)].

Preserved larval endplates are associated with preserved larval leg muscle

In one type of heterochronic mosaic, we injected JHM into one larval leg prior to the commitment pulse of ecdysteroids to prevent the commitment of the tissues in that leg to a pupal fate (see Fig. 1). In some preparations, the contralateral leg was injected with mineral oil to control for effects of the injection itself. The contralateral leg also served as a control for diffusion of JHM and systemic effects of the treatment. This experimental manipulation resulted in a pupa that had one retained larval leg. In some cases, the JHM injection into the leg perturbed normal development, and the animals did not pupate normally. Those animals were not included in this study. In most preparations (n=52), the JHM-injected leg retained larval characteristics that varied in degree from one animal to the next. In some cases, all segments of the JHM-injected leg were larval-like, with larval cuticle, larval sensory hairs, and larval leg muscles (see Fig. 3D). In other cases, there were patches of larval cuticle interspersed with pupal cuticle on one or more leg segments. This variability of larval tissue preservation was likely due to the amount of JHM injected. When all segments of the leg were injected, preservation of the larval features was more uniform within the leg.

Upon dissection, preparations that had some visible degree of juvenilization of the JHM-injected leg were prepared for biocytin labeling of the leg nerves or for anti-synaptotagmin immunohistochemistry. In some preparations, Fluoro-Gold was injected into the legs to reveal the muscles. When FeDe muscles from oil-injected legs or untreated contralateral legs were examined, we found that the larval FeDe muscle degenerated on schedule (right sides in Fig. 3A, B) and by P0 resembled those in normal, unmanipulated animals. In contrast, the JHM-treated leg contained a larval-like FeDe

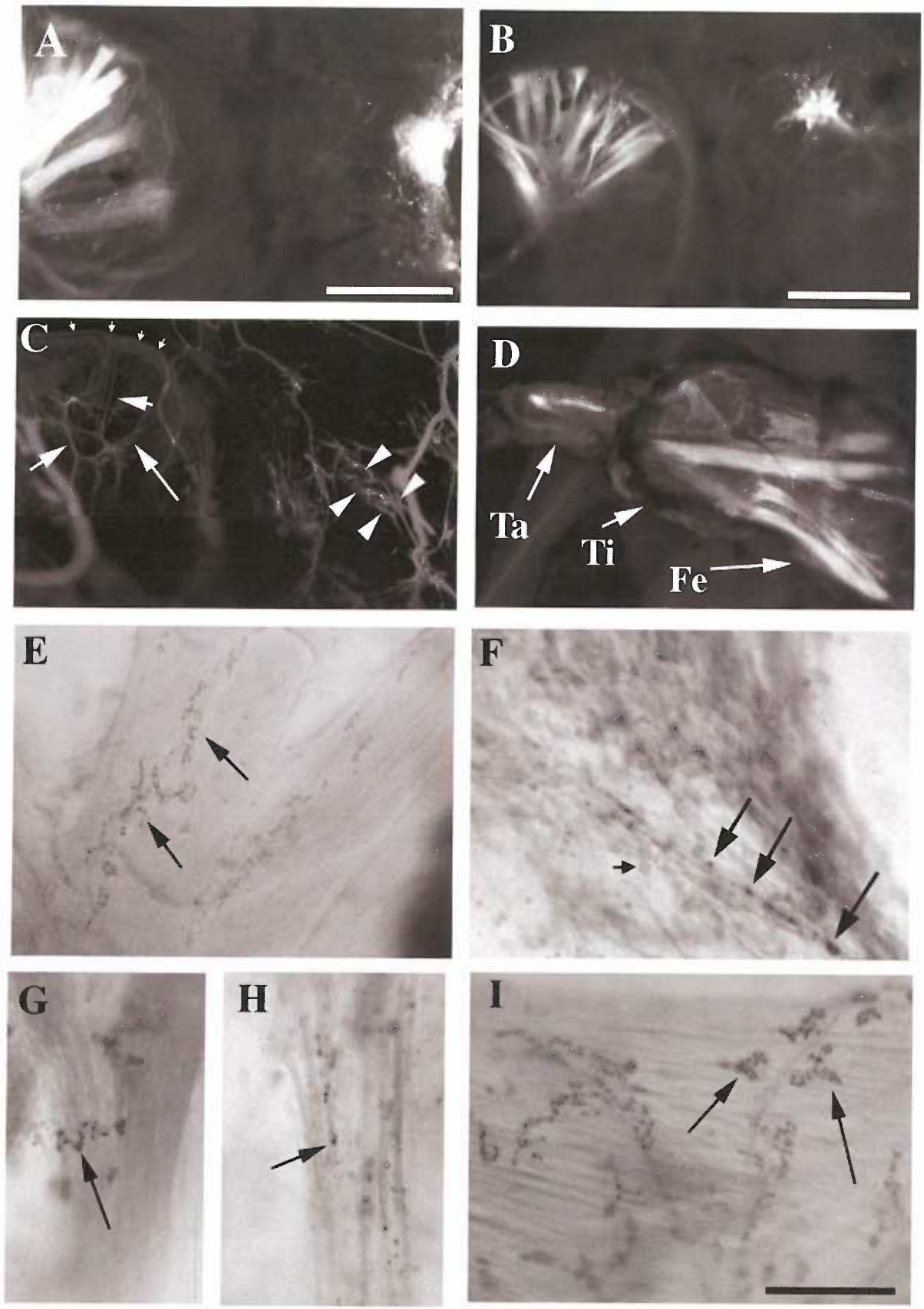

Figure 3

muscle (left sides in Fig. 3A, B). Labeling of the leg nerves with biocytin revealed that in the control leg, the nerve branches had retracted and remained associated with the muscle remnant (arrowheads, Fig. 3C) as in normal P0 animals. On the JHM-treated side, nerve branches had not retracted but rather extended along the length of the larval-like muscle fibers (arrows, Fig. 3C). Anti-synaptotagmin labeling of the control muscles revealed that larval endplates had been lost (Fig. 3F), and the staining pattern was similar to that of unmanipulated P0 FeDe muscle remnants, with few anti-synaptotagmin positive varicosities scattered on the remnant (compare with Fig. 2F). In contrast, anti-synaptotagmin labeling of JHM-treated legs demonstrated that rosette-like endplates were associated with the preserved muscle fibers (Fig. 3E). In some preparations, the JHM treated legs contained leg muscles that appeared only partially preserved, similar to those in a W2 prepupa (left side, Fig. 3B). In others, a gradient of histolysis occurred within a single muscle fiber such that one end appeared larval-like and the other was histolyzed. Rosette-like endplates were associated only with the ends of the muscle fibers that were preserved (compare Fig. 3G and 3H). The more distal leg muscles were more often preserved in their larval-like form than the FeDe muscle (Fig. 3I), possibly because the JHM was contained better within the distal leg segments.

The larval dendrites of the FeDe MN normally undergo dendritic regression during the wandering stage and the first 3 days of the pupal stage such that the entire larval dendritic arbor is lost (Kent and Levine, 1993). At stage P0, intracellular cobalt staining of the FeDe MN in control animals revealed that dendrites in the lateral and dorsal-medial regions of the neuropil had almost completely regressed leaving only a few sparse processes (Fig. 4A), as reported previously (Kent and Levine, 1993). To

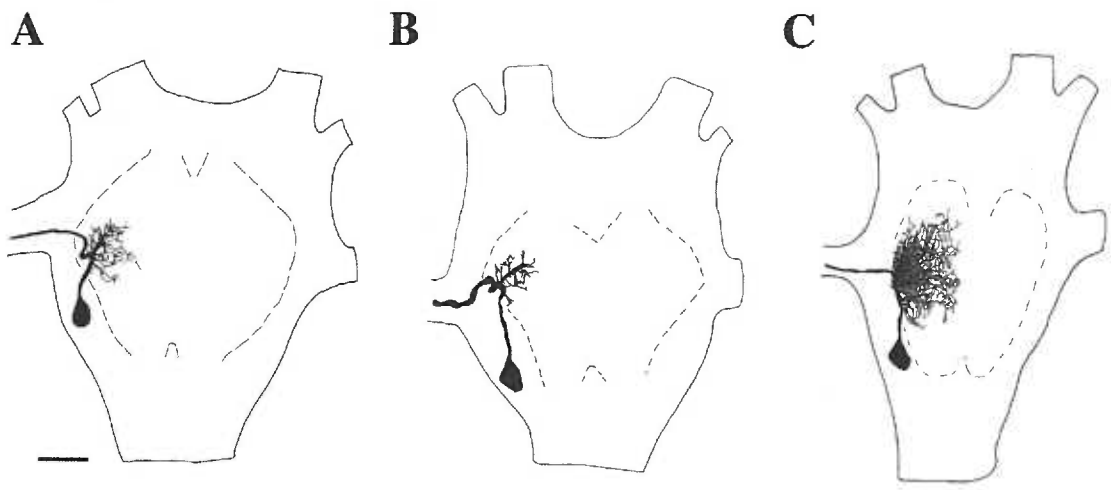

Figure 4. Camera lucida drawings of cobalt-stained FeDe MNs from a normal P0 pupa (A), a mosaic P0 pupa that had JHM injected into the left leg prior to the commitment pulse (B), and a normal fifth-instar larva (C). The FeDe MN shown in (B) innervated the preserved larval leg muscles shown in Fig. 3E. The JHM injection did not prevent regression of larval dendrites. Anterior is up. Scale bar = 100 μm.

determine if JHM treatment of the larval leg and preservation of the larval leg muscle affected dendritic regression, we examined dendritic morphology in a subset of preparations (n=10). Intracellular cobalt staining of the FeDe MN that innervated the JHM-injected leg demonstrated that regression of larval dendrites occurred in all preparations examined (Fig. 4B; compare with dendritic morphology of normal fifth instar larva in 4C), despite retention of larval-like motor terminals (Fig. 3E). The JHM treatment in the periphery, therefore, did not prevent dendritic regression, suggesting that JHM did not reach the CNS in sufficient quantities to interfere with central ecdysteroid actions.

Local loss of endplates is associated with local histolysis of larval leg muscle.

To determine if ecdysteroid action on the CNS is necessary to trigger motor-terminal retraction, we created a second type of heterochronic preparation in which local degeneration of the larval FeDe muscle was triggered in a developmentally-arrested larva. In initial experiments, we established that ligation through the mesothoracic segment on W0 would arrest development, thus preventing degeneration of the FeDe muscle and regression of the FeDe MN's larval dendrites. We ligated larvae on day W0 to eliminate the source of ecdysteroids (the prothoracic glands), which prevented the prepupal peak from occurring. The ligated larvae were maintained for 4 to 6 days, or as long as 11 days. Dissection and processing of FeDe muscles for anti-synaptotagmin immunohistochemistry from ligated larvae revealed that ligation prevented degeneration of the larval FeDe muscle and retraction of FeDe motor terminals. In all preparations examined (n=7), larval leg muscles were still present as was the rosette-like pattern of anti-synaptotagmin immunoreactive endplates (Fig. 5A). Leg muscles and synaptic

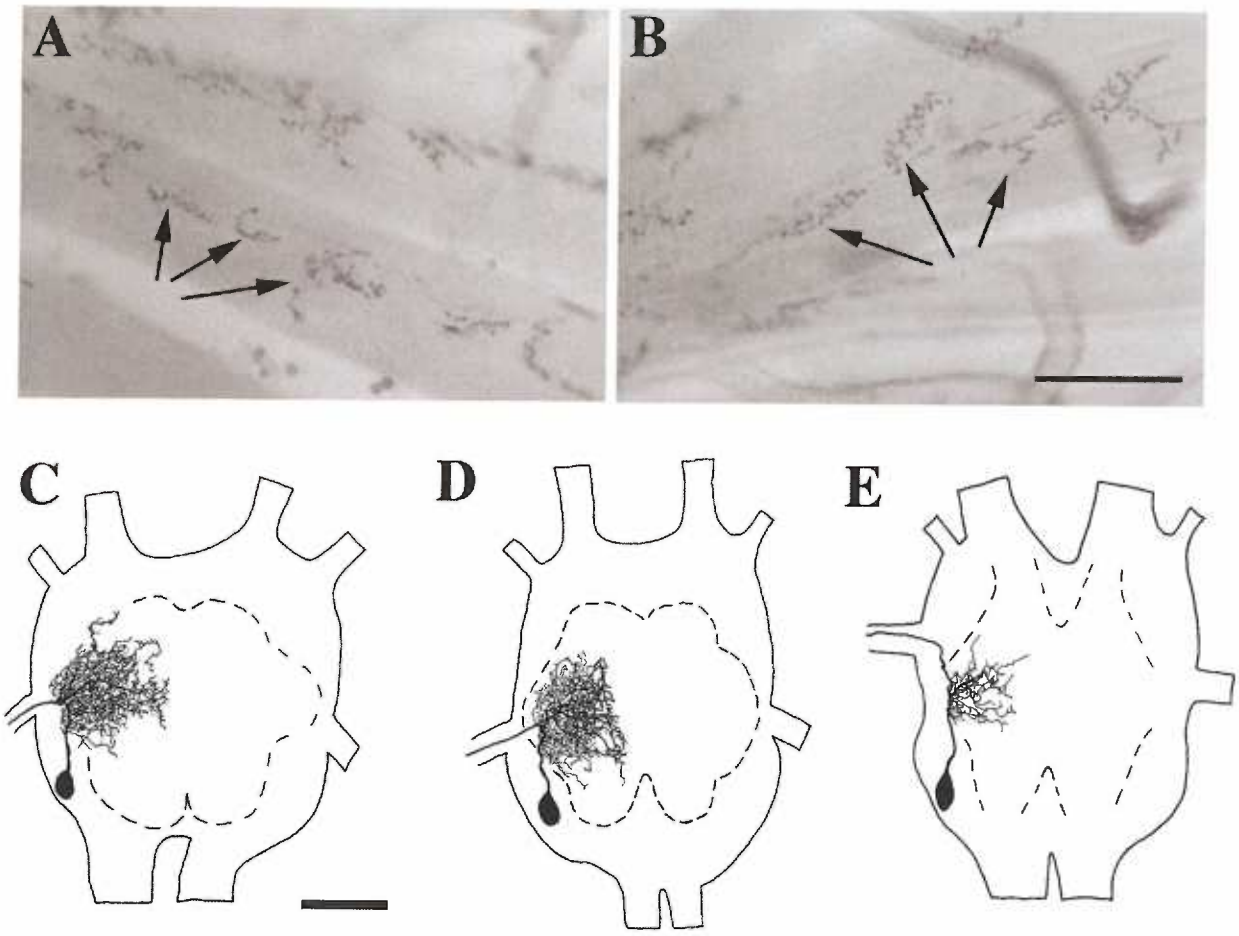

Figure 5. Anti-synaptotagmin labeling of motor terminals on FeDe muscles in a larva 4 days after ligation at W0 (A) and in a normal W0 stage larva (B). The rosette-like arrangement of boutons is preserved in the ligated larvae. (C, D, E) Camera lucida drawings of the FeDe MN in a larva 4 days after ligation at W0 (C), in an unligated W0 stage larva (D), and in a normal W4 stage prepupa (E). Scale bars = 100 μm in (B) [same magnification as in A], and (C) [same magnification as in (D, E)].

boutons were similar to those from unligated control animals at stage W0 (Fig. 5B). In addition, intracellular cobalt staining of the FeDe MN in ligated larvae demonstrated that the regression of larval dendrites was also prevented by ligation at W0 (Fig. 5C; compare with normal W0 in 5D and W4 in 5E).

To trigger the local degeneration of the larval FeDe muscle in another group of developmentally-arrested animals, one leg was injected with RH5992 one or two days after ligation. The contralateral leg was injected with the carrier (DMSO/mineral oil) and served as a control for effects of the injection itself. The contralateral leg also served as an internal control for diffusion of the RH5992 and systemic effects of the treatment. The RH5992 injection initiated development of the treated leg, which could be detected by observing the legs through a dissection microscope. The injected leg ceased to respond to stimulation of the tactile setae on the legs, the larval leg cuticle stiffened and turned brown, and the epidermis often underwent apolysis, retracting away from the overlying cuticle. The treated animals were dissected between 4 and 11 days following injection. The RH5992-treated legs varied such that in some preparations larval leg muscles underwent histolysis and a pupal leg epidermis developed and underwent apolysis (n=7). In other preparations, the RH5992 treated leg muscles showed signs of degeneration but there was no development of a pupal epidermis (n=7). Fluoro-Gold labeling of the muscles in the ligated larvae illustrated the different degrees of muscle degeneration in the RH5992-injected legs by comparison to the larval leg muscle in the contralaleral legs (Fig. 6A and B). In most preparations examined, the RH5992 treated legs contained degenerating muscles that resembled those in W2 - W3 stage prepupae (Fig. 6A and B). Those that progressed further were difficult to evaluate due to the

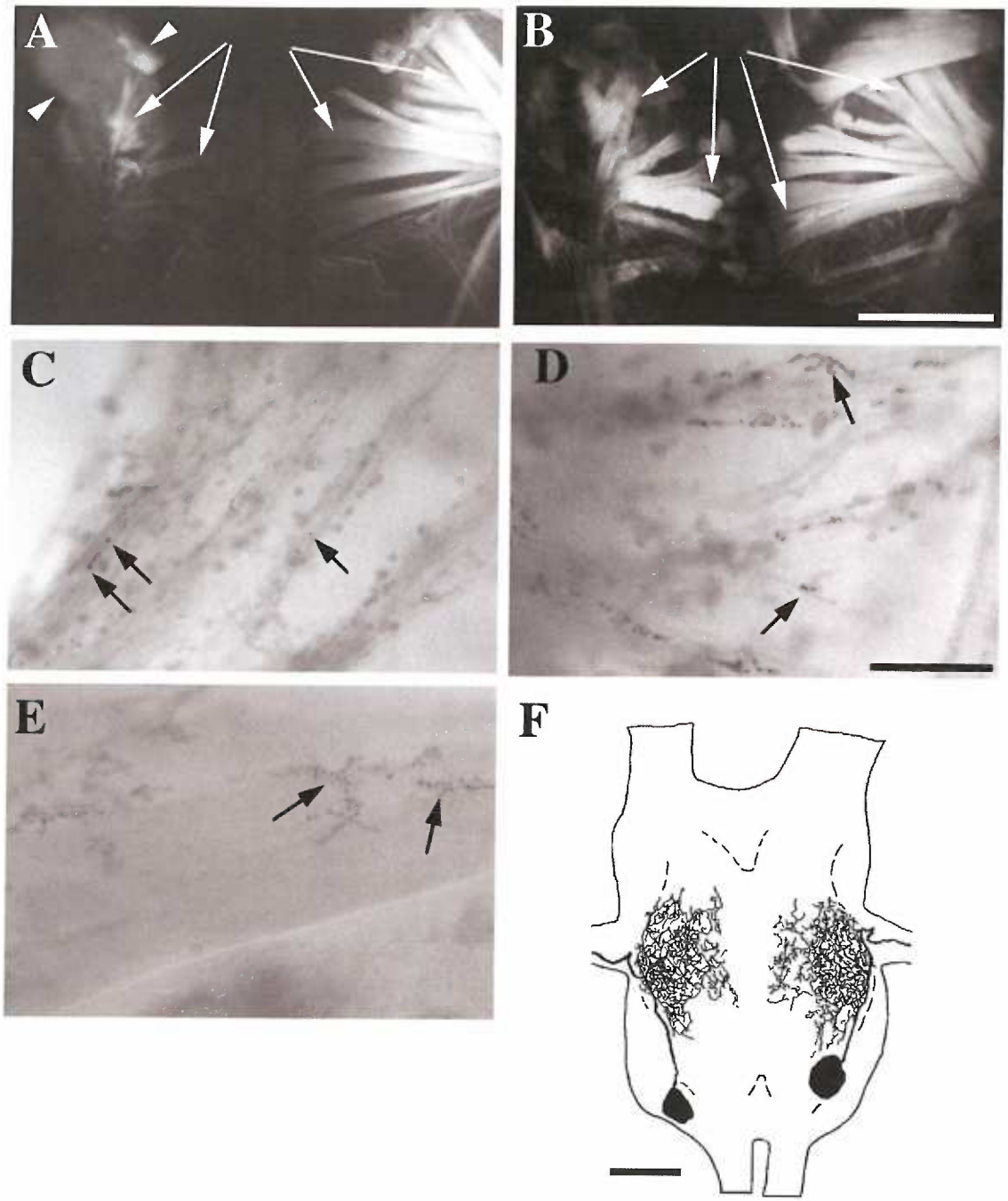

Figure 6

development of adult apodemes (tendons) and the inability of the pupal leg to expand as it normally would at the time of pupation.

Anti-synaptotagmin immunostaining demonstrated that larval endplates were lost on the muscles that had undergone histolysis in response to the RH5992 injection (Fig. 6C), while endplates remained intact on the carrier-injected leg muscles (Fig. 6E). Leg muscles that underwent partial histolysis had endplates that resembled those in normal W2 - W3 prepupae (Fig. 6D; compare with Fig. 3D, E). Intracellular cobalt labeling of the FeDe MN confirmed that although motor terminals were lost, MN dendritic regression had not occurred (n=5; Fig. 6F; compare with Fig. 4C), indicating that RH5992 had not reached the CNS in sufficient quantities to trigger dendritic regression in the CNS.

These results suggest that peripheral actions of ecdysteroids during the pupal peak are sufficient to cause loss of larval endplates and that loss of larval endplates can occur without concomitant regression of central dendrites. These results do not rule out the possibility, however, that central ecdysteroid action during the commitment pulse is necessary for MN terminal retraction during the pupal peak.

Incomplete loss of retracted varicosities is associated with a preserved larval CNS

To determine if motor-terminal retraction and endplate loss require commitment of the MN to a pupal fate, we created a third type of heterochronic mosaic that had a preserved larval ganglion in an otherwise pupal body. We injected JHM into metathoracic ganglia on the first day of the fifth larval instar to prevent commitment of the neurons in the injected ganglion, including the FeDe MN. Control animals were injected with an equivalent amount of mineral oil to account for effects of the injection

alone. In most cases, the CNS injections did not alter the normal time course of development (n=21, oil; n=42, JHM) and animals were sacrificed on the day of pupation. Intracellular cobalt staining was used to evaluate the dendritic morphology of the FeDe MN. In 6/6 cobalt-stained oil-injected preparations, normal regression of the FeDe MN's larval dendrites occurred (Fig. 7A; compare to Fig. 4A). In contrast, in 17/19 JHM-injected preparations, the FeDe MN's larval dendrites did not regress (Fig. 7B; compare to Fig. 4C). When we examined the FeDe muscles from stage P0 animals in which JHM had been injected into the ganglion, we found that the larval FeDe muscles had degenerated normally and an anlagen comparable to that of a normal P0 was present. However, the P0 anlagen from JHM-injected animals seemed to have more anti-synaptotagmin positive varicosities associated with it when compared to oil-injected controls (compare Fig. 7C and D). Although the rosette-like larval endplates were disrupted, clusters of varicosities were located on the muscle remnant in the JHM-treated preparations. In contrast, there were few clusters of varicosities present in the oil-injected control preparations, similar to normal P0 preparations (compare to Fig. 2F). Biocytin staining of the motor nerves did not reveal obvious differences between oil-injected control preparations and JHM-injected preparations, suggesting that retraction of axonal branches had occurred in the JHM preparations (data not shown). Due to the fragile nature of the P0 preparations, we were concerned that biocytin might not reliably stain all nerve processes. In the remaining preparations, therefore, we used an antibody against GPI-linked *Manduca* Fasciclin II (GPI-MFasII), which labels the glial sheath surrounding the peripheral nerves and serves as a useful marker for nerve branches (Knittel et al., 2001).

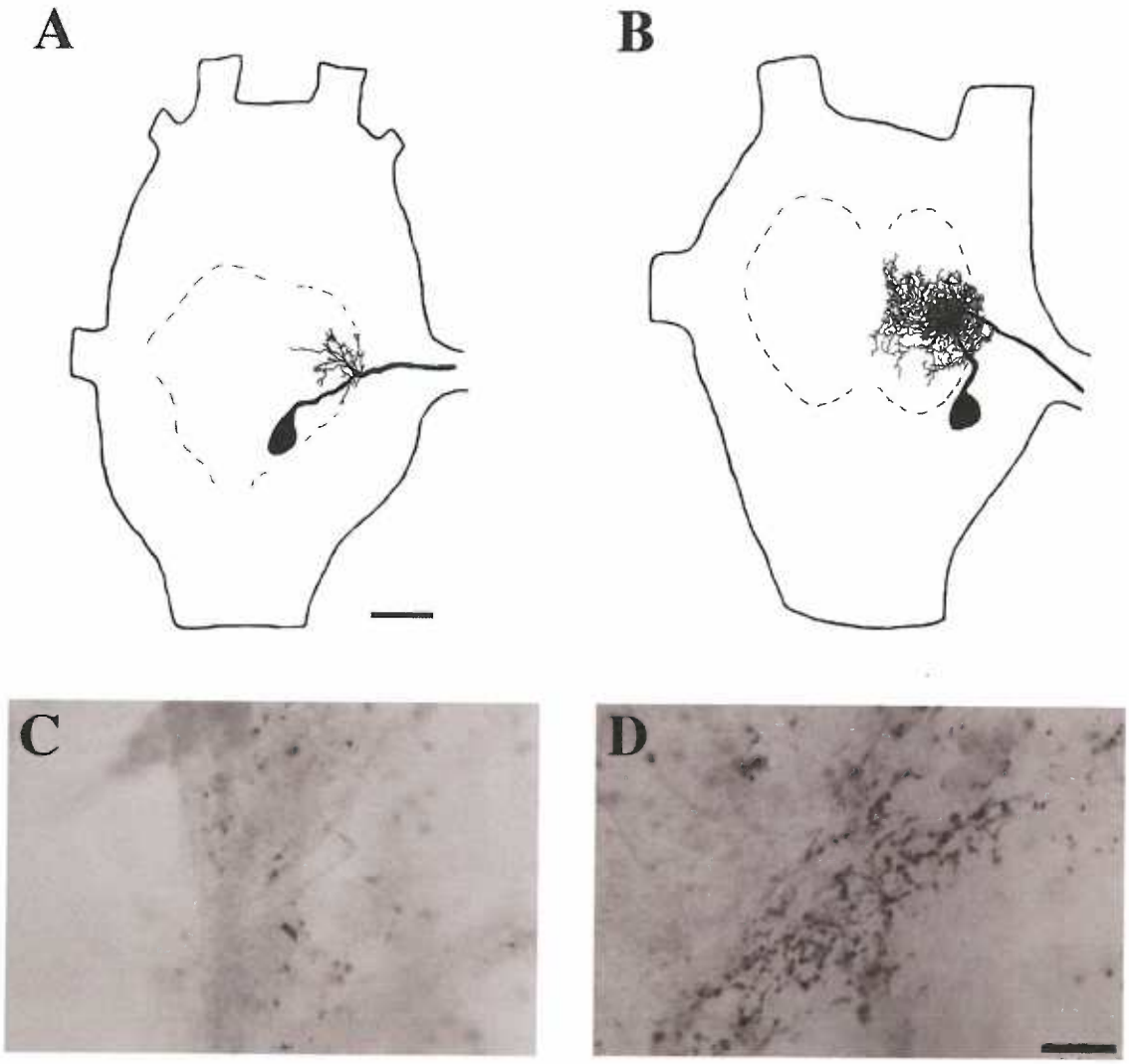

Figure 7. Dendrites and terminals of FeDe MNs from control and heterochronic mosaic insects that received injections of oil (A, C) or JHM (B, D) in the metathoracic ganglion prior to the commitment pulse of ecdysteroids. (A, B) Camera lucida drawings of FeDe MNs labeled by intracellular injection of hexamine cobaltic chloride at stage P0. Injection of JHM prevented the regression of larval dendrites (B), while the oil injection had no apparent effect on dendritic regression. The FeDe MN in (B) innervated the FeDe muscle shown in Fig. 8D and E. (C, D) Anti-synaptotagmin labeling of FeDe muscles and motor terminals at stage P0. FeDe muscles from animals injected with JHM in the metathoracic ganglion prior to the commitment pulse of ecdysteroids have more anti-synaptotagmin immunoreactivity associated with them (D) when compared to oil-injected controls (C). Scale bars = 100 μm in (A) [same as magnification in (B)] and (D) [same as magnification in (C)].

To obtain a better view of the relationship between the anti-synaptotagmin labeled varicosities and the retracted nerve terminals, we used confocal microscopy to visualize anti-synaptotagmin and anti-GPI-MFasII labeling in the same preparations (n=4, oil; n=8, JHM). Among these, the JHM-injected preparations displayed more anti-synaptotagmin labeling associated with the muscle anlagen than did the oil-injected control (compare Fig. 8B with 8E and 8C with 8F). Differences in anti-GPI-MFasII labeling were not obvious and the staining pattern varied among both experimental and control preparations. Both JHM- and oil-injected preparations had thick anti-GPI-labeled processes associated with the muscle anlagen (compare Fig. 8A with 8D and 8C with 8F). In both types of preparations, anti-synaptotagmin labeling was primarily associated with the anti-GPI labeled processes, but in the oil-treated preparations most of the anti-GPI-labeled processes had sparse anti-synaptotagmin labeled varicosities (Fig. 8C). In contrast, in the JHM-treated preparations, the anti-GPI-labeled processes contained numerous anti-synaptotagmin labeled varicosities (Fig. 8F). Thus, the rosette-like structure of the larval endplates was disrupted and the retraction of fine axon branches occurred in animals with JHM-injected ganglia, but the final loss of anti-synaptotagmin positive varicosities did not occur. This aspect of endplate loss, therefore, appears to require pupal commitment of the FeDe MN.

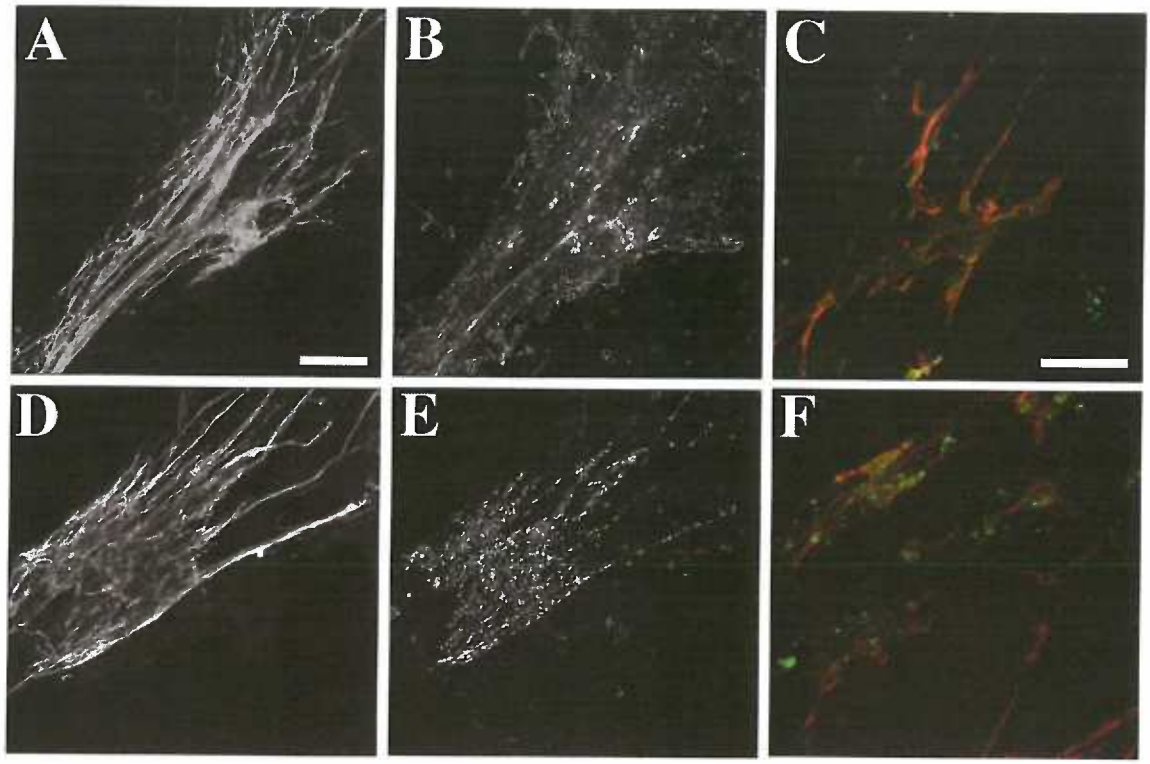

Figure 8. Confocal micrographs of FeDe muscles from animals that had either oil (A, B, C) or JHM (D, E, F) injected into the metathoracic ganglion prior to the commitment pulse. (A, B, D, E) Low magnification images of preparations double labeled with anti-GPI-MFas II (A, D) and anti-synaptotagmin (B, E). FeDe muscles from animals injected with JHM have more anti-synaptotagmin positive varicosities when compared to oil-injected controls. However, motor-terminal retraction, as visualized using anti-GPI-MFas II labeling, has occurred in both JHM and oil-injected preparations. (C, F) High magnification merged images of preparations double labeled with anti-synaptotagmin (green) and anti-GPI-MFas II (red). Scale bar = 100 μm in (C) [same as magnification in (A, B, D-F)].

DISCUSSION

We have manipulated the hormonal environment of the FeDe MN such that its dendrites and soma were exposed to a different hormonal milieu than its motor terminals and target muscle. These manipulations produced several types of heterochronic mosaic insects in which development of the CNS and development of the periphery were out of synchrony, allowing us to test the roles of peripheral and central actions of ecdysteroids on MN remodeling. Our results indicate that dendritic regression is not affected by the hormonal milieu of the leg, or the developmental stage of the leg or leg muscle, whereas motor-terminal retraction is affected by these factors. Ecdysteroid action in the periphery appears sufficient to trigger motor-terminal retraction, but the complete elimination of retracted synaptic varicosities appears to require central ecdysteroid action during the commitment pulse.

Remodeling of the FeDe MN and FeDe muscle during metamorphosis: role of the prepupal peak

Dendritic regression and retraction of FeDe motor terminals occur over the same time course as degeneration of the larval FeDe muscle (Kent and Levine, 1993; Knittel et al., 2001), and all begin during the rise in the prepupal peak of ecdysteroids, suggesting that the rise in the ecdysteroid titer triggers these changes. In this study, ligation of larvae on W0 prevented the regressive changes in the FeDe MN and FeDe muscle (Fig. 5), supporting the idea that the prepupal ecdysteroids are the trigger. In studies on the principal planta retractor MN (PPR) and its target muscle (PPRM), dendritic regression and muscle degeneration have been shown to follow a similar time course. Ligation and infusion experiments established that the rising phase of the prepupal peak of the

ecdysteroids was the proximate trigger for the changes in both the muscle and the MN (Weeks and Truman, 1985). A similar coordination of muscle degeneration with motor-terminal retraction has been reported for the abdominal muscle DEO1 and MN-12, except that the falling phase of the prepupal peak is the proximate trigger (Hegstrom, 1996a; Hegstrom, 1996b).

Role of cell-cell interactions

Muscle histolysis at the end of larval life appears to be triggered by a direct action of the ecdysteroids on the muscle fibers, rather than by ecdysteroid action on the innervating MN. Experiments in which PPRM was denervated indicated that the loss of innervation was not sufficient for muscle degeneration nor was maintained innervation necessary (Weeks and Truman, 1985). Similarly, transection of the nerve to the FeDe muscle during larval life had no effect on FeDe degeneration (L.M. Knittel, unpublished observations; Consoulas and Levine, 1997). *In vitro* studies of muscles in the fly, *Calliphora erythrocephala,* indicate that a muscle's response to ecdysteroids is intrinsic. Larval muscles that were destined to die during metamorphosis degenerated in culture when exposed to 20E, whereas those that were destined to survive did not degenerate upon exposure to 20E (Zachary and Hoffmann, 1980). In our experiments, local JHM application prior to the commitment pulse of ecdysteroids preserved the larval leg and leg muscle (Fig. 3), consistent with the idea that central actions of ecdysteroids do not control muscle degeneration. This is the first report of a larval muscle being preserved by local JHM treatment, although previous studies have described the appearance of regrown larval muscles in the adult as a result of JHM treatment during the early pupal stage (Truman and Reiss, 1995). Exposure of the larval FeDe muscle to JHM during the

commitment pulse presumably prevented pupal reprogramming of the muscle and hence prevented its degeneration in response to the prepupal peak. In some cases, JHM-treated leg muscle fibers appeared to be preserved at one end but histolyzed at the other end. Since these muscles are multinucleate, a likely explanation is that JHM prevented reprogramming within a subset of nuclei that were closest to a droplet of oil containing JHM. More distal nuclei, presumably, underwent reprogramming and responded to prepupal ecdysteroids, initiating local muscle histolysis.

Dendritic regression at the end of larval life appears to be triggered by a direct action of the ecdysteroids on the CNS, rather than by ecdysteroid action on the target muscle or sensory neurons (Jacobs, 1990; Weeks, 1987; Weeks and Truman, 1985). PPR undergoes dendritic regression on schedule even if the motor nerve has been transected or its target muscle has been surgically eliminated (Weeks and Truman, 1985). Muscle death and dendritic regression also have different endocrine requirements, such that carefully timed ligations can prevent muscle death while dendritic regression proceeds (Weeks, 1987). Our results also indicate that dendritic regression proceeds on schedule, despite the preservation of a larval leg and leg muscle (Fig. 4). In contrast, injection of JHM into the metathoracic ganglion prevented dendritic regression without preventing muscle degeneration (Fig. 7). Thus, our results support and extend previous findings that dendritic regression is triggered by central ecdysteroid action and is not modulated by the developmental stage of the target.

In contrast to dendritic regression, motor-terminal retraction appears to be influenced by peripheral ecdysteroid-induced changes. In a previous study, Truman and Reiss (1995) used local JHM treatments of muscle DEO1 during the early pupal stage to

prevent growth of adult muscle DE5, which was replaced by a re-grown larval-like DEO1. Although endplate loss occurred normally on JHM-treated muscles, these re-grown larval-like muscles were associated with larval axonal branches, suggesting that axonal pruning was influenced by the target. In the current study, we treated the larval FeDe muscle with JHM prior to the commitment pulse to prevent the FeDe from degenerating in response to the prepupal peak of ecdysteroids. As described above, dendritic regression occurred on schedule indicating that ecdysteroid action on the CNS had occurred, but rosette-like boutons remained associated with the preserved larval leg muscle (Fig. 3E). Therefore, endplate loss as well as axonal pruning was prevented by preserving the larval target muscle. This result supports the idea that interactions with preserved peripheral tissues can modulate the peripheral response of the MN to central ecdysteroid action. Although we have no evidence that non-genomic mechanisms underlie motor-terminal retraction in *Manduca*, our results are also consistent with direct effects of JHM on the terminals. In any case, our results indicate that central ecdysteroid action is not sufficient to induce motor-terminal retraction. An alternate explanation is that dendritic regression and terminal retraction have different endocrine requirements. A low level of JHM reaching the CNS might have been sufficient to interfere with ecdysteroid-induced motor-terminal retraction, but not dendritic regression. However, the partial preservation of larval muscle in some preparations and the association of rosette-like boutons only with preserved fibers argue against that explanation. If a small amount of JHM had reached the CNS and interfered with central ecdysteroid-induced terminal retraction, it should have produced a uniform response among all terminals of the FeDe MN.

Results from other experiments suggest that central ecdysteroid action during the prepupal peak might not even be necessary for motor-terminal retraction. When RH5992 was applied to one metathoracic leg of a larva ligated prior to the prepupal peak of ecdysteroids, the treated leg underwent the characteristic developmental processes that normally precede pupation, including muscle histolysis and loss of larval endplates (Fig. 6C). In contrast, the contralateral leg remained morphologically and functionally larval, and larval muscles and rosette-like endplates were retained (Fig. 6E). These results suggest that a local action of ecdysteroids on peripheral tissues, such as the FeDe muscle, controls retraction of nerve terminals independent of the hormonal milieu of the MN soma. Similar results have been reported for muscle DEO1, but those experiments did not include studies on the CNS of hormonally-manipulated animals to verify that the CNS was unaffected by the hormonal manipulations (Hegstrom, 1996b). Our results revealed that peripheral RH5992 treatments did not trigger the FeDe dendrites to regress even though it caused the FeDe muscle to degenerate and the motor terminals to retract. Therefore, the RH5992 treatment in the periphery did not appear to affect the CNS. An alternate explanation is that dendritic regression and endplate loss have different endocrine requirements. Low levels of RH5992 reaching the CNS might have been sufficient to trigger endplate loss but not dendritic regression. An argument against that explanation is provided by the results of Hegstrom and Truman (1996b). In that study, topical application of RH5992 induced local histolysis of muscle DEO1, such that endplate loss was associated with regions of a single muscle fiber that underwent histolysis while endplates were associated with regions that did not undergo histolysis. If low levels of RH5992 reaching the CNS were responsible for triggering endplate loss,

uniform loss of boutons would be expected. Nevertheless, they did not rule out the possibility that central RH5992 (or ecdysteroid) action was necessary to make the MN competent to respond to signals from the peripheral tissues.

Together, our results and those of Truman and colleagues suggest that signals from degenerating target muscle influence motor-terminal retraction. However, not all MNs respond to muscle degeneration with a similar coincidence. For example, in the prothoracic leg, larval-like presynaptic terminals of the TiExt MN were still present on W3 after degeneration of the target muscle (Consoulas et al., 1996). Moreover, retraction of MN terminals from the same MN may not be coincident with muscle degeneration at another stage of life. For example, the adult target muscle of MN-12 degenerates after adult emergence, but MN terminals remain associated with the degenerated muscle and maintain presynaptic terminals (DeLorme and Mesce, 1999). Therefore, the signals that trigger motor-terminal retraction are MN- and stage-specific. Commitment or competence of the MN to respond to peripheral signals may depend upon the expression of specific receptors, intracellular signaling molecules, or downstream effector molecules, which may be stage dependent or unique to particular MNs.

Role of the commitment pulse of ecdysteroids

The role of the commitment pulse is to allow the genetic switch from the expression of larval genes to the expression of pupal genes (Riddiford, 1996b). For most tissues, the commitment pulse has no overt effect, but alters the response of the tissues to the subsequent prepupal peak of ecdysteroids. For muscles such as FeDe and PPRM, the commitment pulse has no overt effect on muscle degeneration (Fig. 2C; Weeks and Truman, 1985; Weeks and Truman, 1986), but renders the muscle more responsive to the

prepupal peak of ecdysteroids. Similarly, the commitment pulse has no effect on dendritic regression, but allows the MN to respond to the prepupal peak of ecdysteroids by undergoing dendritic regression (Weeks and Truman, 1986). The commitment pulse also renders the MN insensitive to JH during the prepupal peak (Weeks and Truman, 1986). In the current study, treatment of larval ganglia with JHM prior to the commitment pulse preserved the larval ganglia without affecting the hormonal environment of the leg. This hormonal manipulation provided an additional method for determining whether ecdysteroid action on the CNS was necessary for motor-terminal retraction, or if interactions with degenerating target muscle were sufficient. Treatment of larval ganglia with JHM prior to the commitment pulse prevented regression of the FeDe MN's larval dendrites, but it did not prevent normal degeneration of the larval FeDe muscle, nor did it prevent the retraction of larval synaptic terminals (Fig. 7). However, exposure of the larval ganglia to JHM prior to the commitment pulse prevented the elimination of synaptotagmin-positive varicosities from the retracted terminals (Fig. 8). This result suggests that although some aspects of motor-terminal retraction do not require ecdysteroid action on the CNS, the FeDe MN must nonetheless experience the commitment pulse of ecdysteroids in order to respond completely to signals from the periphery. Once a MN becomes pupally committed, however, interactions with degenerating target muscle appear to be sufficient to trigger all aspects of motor-terminal retraction.

Mechanisms underlying motor-terminal retraction

The mechanisms involved in the dismantling of synapses are not known. In vertebrates, synapse elimination occurs during early postnatal life and following

reinnervation as a result of injury. Synapse elimination involves synaptic competition between the motoneurons innervating each endplate (Lichtman and Colman, 2000), but several aspects of this process are similar to those occurring in *Manduca* during remodeling. For example, in vertebrates, as in *Manduca*, local interactions at each endplate appear to influence the retraction of individual branches (Keller-Peck et al., 2001). Anatomical similarities also suggest common mechanisms. Terminal glial cells retract their processes during motor-terminal retraction in vertebrates (Culican et al., 1998) and in *Manduca* (Rheuben, 1992), suggesting a role for glia in both cases. In vertebrates, retracting axonal branches end in enlarged varicosities termed "retraction bulbs" (Riley, 1977), which often appear to be unattached to the junction, suggesting that loss of adhesion may precede the withdrawal of axonal processes (Gan and Lichtman, 1998). In *Manduca*, retracting terminals also form enlarged varicosities, but some portions of the nerve terminal may rupture while the remainder is engulfed by glial cells (Rheuben, 1992). Changes in adhesive interactions between the muscle and glia and/or muscle and nerve, however, could trigger such changes in the terminals. In this regard, it is interesting that the transmembrane isoform of the homophilic adhesion molecule *Manduca* Fas II is upregulated in the FeDe MN during motor-terminal retraction (Knittel et al., 2001). Glial cells express the GPI-linked isoform of *Manduca* Fas II, providing for possible homophilic interactions between the MN and glia that could underlie some aspects of motor-terminal retraction.

Studies of the subcellular changes underlying regression of terminals in the vertebrate CNS might also be relevant to the mechanisms underlying motor-terminal remodeling in *Manduca*. In the CNS of adult rats, loss of thalamic target neurons leads to

retraction and resorption of afferent axonal processes that normally project to those targets (Marty and Peschanski, 1994). The retracting neuronal processes consist of varicosities containing an accumulation of synaptic proteins (e.g. synaptophysin) and organelles, suggesting that the cell body of the neuron may still be supplying the terminals with the machinery for synaptic transmission even though the target has been lost (Marty and Peschanski, 1994). However, changes in the accumulation of organelles associated with membrane recycling and degradation occur, such that synaptic vesicles decrease and coated vesicles and multivesicular bodies increase. Terminal varicosities often contain lysosome-like structures, suggesting that "local autophagia" may be the mechanism by which axon terminals are resorbed (Marty and Peschanski, 1994).

In *Manduca*, retracting FeDe MN processes also contain varicosities that are anti-synaptotagmin positive, but most of the anti-synaptotagmin immunoreactivity is lost between W3 and P0 (Knittel et al., 2001). Our results from JHM injections into the larval ganglia prior to the commitment pulse indicated that the retracted motor terminals retained more anti-synaptotagmin immunoreactive varicosities than oil-injected controls. Thus, some final degradative aspect of motor-terminal retraction failed to occur in response to peripheral cues when the MN was not committed. Interfering with the commitment pulse may have prevented a pupal-specific response to peripheral signals, such as the transport of lysosomes to the motor terminals that might be involved in an "autophagic" dismantling of retracted larval motor terminals.

Uncoupling of dendritic regression and motor-terminal retraction

Results from our heterochronic mosaic insects indicate that, for the FeDe MN, dendritic regression and motor-terminal retraction can occur independently of each other.

A similar ability to separate specific aspects of MN remodeling has been described for PPR (Weeks and Truman, 1985). PPR's dendritic regression and subsequent cell death can be experimentally uncoupled, indicating that the two events have different endocrine requirements and that dendritic regression is not simply a symptom of impending cell death (Weeks, 1987). These findings also support a model of "compartmental neurodegeneration" that has been proposed to underlie Wallerian degeneration in the mouse (Gillingwater and Ribchester, 2001). In that model, synaptic degeneration occurs by a separate mechanism from axonal or cell body degeneration. The experimental uncoupling of the FeDe MN's dendritic regression and motor-terminal retraction in our experiments suggests that regressive events at opposite ends of the MN are regulated by different signals. In the periphery, motor-terminal retraction appears to be a response to ecdysteroid actions on target muscle or other peripheral tissues. Although we currently have no evidence for non-genomic actions of ecdysteroids in *Manduca*, our results are also consistent with direct, non-genomic effects of ecdysteroids on the motor terminals themselves. In the CNS, dendritic regression might be a direct response to ecdysteroid action on the FeDe MN, though it could also be an indirect response to ecdysteroid action on other central cells. Experiments manipulating central glia and interneurons independent of the FeDe MN will be required to determine the role of cell-cell interactions in dendritic remodeling.

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CHAPTER 3

Remodeling of an Identified Motoneuron During Metamorphosis: Central and Peripheral Actions of Ecdysteroids During Growth of Dendrites and Motor Terminals

SUMMARY

During metamorphosis of the tobacco hawkmoth *Manduca sexta*, the femoral depressor motoneuron (FeDe MN), undergoes remodeling of its dendrites and motor terminals. This remodeling is mediated by the same hormones that control metamorphosis: the ecdysteroids and juvenile hormone (JH). In this study, I have asked whether growth and differentiation of adult motor terminals is caused by central or peripheral actions of the ecdysteroids. I used a JH mimic to manipulate the hormonal environment of the FeDe MN soma without affecting the peripheral environment. My results suggest that peripheral actions of ecdysteroids are sufficient to promote motor-terminal elongation and differentiation. My results also demonstrate that adult dendritic growth and motor-terminal growth can be experimentally uncoupled, suggesting that they are regulated differently.

INTRODUCTION

Insects that go through a complete metamorphosis undergo a hormonally-mediated restructuring of their neuromuscular system. Larval muscles that are not needed for pupal and adult behaviors die, and their motoneurons (MNs) either die (Weeks and Truman, 1985) or are remodeled to innervate new target muscles (Hegstrom, 1996b; Weeks and Ernst-Utzschneider, 1989). Remodeled MNs undergo regression of larval dendritic arbors and retraction of larval motor terminals before sprouting new adult dendritic arbors and motor terminals. The insect steroid hormone 20-hydroxyecdysone (20E) has been shown to control the loss of larval processes (Hegstrom, 1996b; Truman and Reiss, 1995; Weeks and Truman, 1986) and the growth of adult processes (Truman and Reiss, 1988; Truman and Reiss, 1995; Weeks and Ernst-Utzschneider, 1989). MN cell bodies and muscles are both targets of ecdysteroid action (Hegstrom et al., 1998; Truman et al., 1994), so ecdysteroids could influence the remodeling of processes by either central or peripheral actions. The goal of the current study was to determine the influences of central and peripheral ecdysteroid action on the growth and differentiation of adult motor terminals.

Metamorphosis of *Manduca* is controlled by the ecdysteroids (primarily by 20E) and by juvenile hormone (JH; reviewed by Riddiford, 1996b). During larval life, the JH titer remains high, and a pulse of ecdysteroids in the presence of JH triggers a molt to the next larval instar. During the fifth and final instar, a rise in the ecdysteroid titer occurs in the absence of JH for the first time, and this "commitment pulse" reprograms larval tissues to a pupal fate. A second larger rise in the ecdysteroid titer called the "prepupal peak" triggers the pupal molt, while the presence of JH in this titer prevents the

precocious development of the imaginal discs (Kiguchi and Riddiford, 1978). A final sustained rise in the ecdysteroid titer called the "pupal peak" occurs in the absence of JH, and this pupal ecdysteroid titer directs development of the moth (Bollenbacher et al., 1981).

The remodeling of neuromuscular systems that occurs during metamorphosis is also controlled by the ecdysteroids, acting both centrally and peripherally. Regression of larval dendrites is controlled centrally by the prepupal peak of ecdysteroids (Knittel and Kent, 2002; Weeks and Truman, 1985), and growth of adult dendrites is controlled by the pupal peak of ecdysteroids (Truman and Reiss, 1988; Weeks and Ernst-Utzschneider, 1989). Retraction of larval motor terminals is controlled peripherally by the prepupal peak of ecdysteroids (Hegstrom, 1996b; Knittel and Kent, 2002), although the MN cell body must experience the commitment pulse of ecdysteroids in order to respond fully to peripheral cues (Knittel and Kent, 2002). In contrast, elongation of adult motor terminals has been suggested to depend on a central action of the ecdysteroids, while differentiation of adult synaptic boutons seems to depend on a peripheral action (Truman and Reiss, 1995).

JH can prevent adult differentiation of remodeled MNs if it is present early during the pupal peak of ecdysteroids. Systemic application of JH prevents the growth of adult dendrites (Truman and Reiss, 1988) and the elongation of adult motor terminals (Truman and Reiss, 1995). Local application of a juvenile hormone mimic (JHM) to a developing adult muscle has been shown to prevent the formation of adult endplates but not the elongation of motor terminals across the muscle (Truman and Reiss, 1995). Together, these results suggest that central ecdysteroid action is sufficient for elongation of motor-

terminals. Whether peripheral ecdysteroid action might also be sufficient for motor-terminal elongation has not been examined. I tested this hypothesis by using a novel heterochronic mosaic in which JHM was applied locally to the CNS rather than to the muscle. In this way, I could ask if peripheral ecdysteroid exposure is sufficient for motor-terminal elongation and differentiation in the absence of central ecdysteroid action.

My results indicate that peripheral ecdysteroid action is sufficient to promote elongation and differentiation of adult motor terminals. Local exposure of the CNS to JHM prior to the pupal peak of ecdysteroids interfered with the growth of adult dendritic processes but did not affect growth of motor terminals. Therefore, adult dendritic changes can be uncoupled from motor-terminal growth, indicating that each is regulated independent of the other. In addition, local exposure of the CNS prior to or during the commitment pulse of ecdysteroids delayed dendritic regression but did not interfere with motor-terminal growth. Finally, my results demonstrate that MNs must undergo dendritic regression before undergoing dendritic growth. A preliminary account of these results has been reported in abstract form (Wilson and Kent, 1998).

METHODS

Animals

Manduca sexta (Lepidoptera: Sphingidae) were obtained from a laboratory culture reared on an artificial diet (Bell and Joachim, 1976) and a long-day photoperiod regimen (17:7 h light/dark) at 26 ^{o}C and approximately 60% relative humidity. Developmental stages were monitored by both chronological and morphological criteria (Nijhout and Williams, 1974; Reinecke et al., 1980; Tolbert et al., 1983; Truman and Riddiford, 1974; Truman et al., 1980). Days during the fifth instar were denoted as L1, L2, etc., and W0 stage larvae were identified by dorsal vessel exposure. P0 designates the day of pupation. Animals maintained under our rearing conditions typically pupated on day 4 or 5 after the onset of wandering and eclosed as moths 18 days after pupation.

Intraganglionic Injections

A juvenile hormone mimic [(JHM) S-methoprene (1μg/ml in mineral oil; gift of Sandoz, Inc.)] was applied to larval ganglia at stage L1, L3, or W0. The goal of these experiments was to have JHM present during the pupal peak of ecdysteroids. However, stage W0 was the latest time point during development that the surgery could be successfully performed, since at later time points the ensuing molt to the pupal stage would have prevented surgical manipulations. Larvae were anesthetized by immersion in ice water or by exposure to CO_2. Larvae were immobilized in a sylgard-coated petri dish, immersed in *Manduca* saline (Weevers, 1966) and an incision along the ventral midline directly above the metathoracic ganglion was made. The metathoracic ganglion was lifted onto a platform made from a pair of forceps and impaled with a microelectrode filled with mineral oil or JHM. About 0.2-0.3 μl of oil or JHM was pressure injected into

the ganglion using a Pneumatic Picopump (WPI). A few crystals of phenylthiourea (PTU; Sigma) were inserted into the wound to prevent oxidation of the hemolymph. The wound was sutured closed using surgical silk and sealed with Vetbond (3M), and the larvae were returned to the colony and allowed to develop until they became moths.

Visualization of Adult Dendrites

The metathoracic FeDe MN was retrogradely labeled by injecting rhodamine dextran lysine (RDL; 3000 MW; Molecular Probes) into the larval femoral depressor muscle. After adult emergence, the pterothoracic ganglion was dissected out, desheathed, and pinned in a sylgard-coated dish. The RDL-labeled cell was located using an Olympus BHJM compound microscope equipped with epifluorescence. The MN was impaled with a glass microelectrode that had a tip resistance of 20-40 MΩ when filled with a solution of hexamine-cobaltic chloride (Sigma;100 mM in 200 mM KAc). Following a 20 minute injection period with 10-15 nA of positive current, the cobalt was precipitated as cobalt sulfide, and the ganglion was fixed in Carnoy's solution (Humason, 1979). The preparations were subsequently processed according to modifications of the Timm's silver intensification method (Bacon and Altman, 1977), dehydrated and cleared with methyl salicylate, and mounted in Canada Balsam (Fisher Scientific).

Quantitation of Dendritic Extent

Drawings of dendrites from individually filled motoneurons were made using a *camera lucida* attachment to a Zeiss compound microscope. In some cases, it was not possible to draw the FeDe MN's dendritic arbor because the staining reaction had made the preparation too dark to discern the fine dendritic processes. Some preparations had

successful cobalt fills of both metathoracic FeDe MNs, and in these cases both MN's dendritic extent were included in the analysis. Drawings were digitized using an Epson 640 Perfection scanner, and NIH Image software was used to count the number of pixels that make up the dendrites. Due to the effect of JHM injections on ganglionic fusion, dendrite counts could not be normalized to the percentage of neuropil covered. Means, errors, and significance were generated using Statview software (version 4.0). A student's one-tailed t test was used to determine the significance of differences between means. P values were significant when less then 0.05.

Nerve-Terminal Staining

To visualize the adult motor terminals, we used a *Drosophila* anti-synaptotagmin antibody (Dsyt2; generously provided by J.T. Littleton and H.J. Bellen), as described previously (Knittel et al., 2001). Briefly, legs were dissected and fixed overnight in 4% paraformaldehyde. Anti-synaptotagmin was used at 1:1000 in phosphate buffered saline + 0.2% Triton X-100 and 5% normal goat serum. Leg muscles were processed for immunohistochemistry using either a biotinylated goat-anti-rabbit secondary antibody and an elite ABC kit (Vector Labs) with DAB as the substrate, or a Cy-5 conjugated goat-anti-rabbit secondary antibody.

Confocal Microscopy

Images were obtained using a BioRad Radiance 2100 laser scanning confocal microscope attached to a Nikon Eclipse E800 fluorescent microscope fitted with 3 lasers: red diode, argon ion, and helium neon. The three lasers generated excitation lines at 488, 514, 543, 568, 633 and 647 nm. Optical sections 1 μm apart were recorded through the

depth of whole-mount preparations. Three-dimensional images were reconstructed using Confocal Assistant software (version 4.02; Todd Clark).

Quantitation of Motor-Terminal Growth

For animals that received intraganglionic injections on day W0, the total length of adult endplates was measured to determine if elongation of nerve terminals had occurred normally. During metamorphic remodeling of the leg neuromuscular system, the nerve retains contact with the central region of the muscle anlagen (Consoulas et al., 1996; Knittel et al., 2001). Therefore, an effect on motor-terminal elongation would most likely be detected at the distal ends of muscle fibers. For the JHM-injected animals, FeDe muscles were dissected only from preparations that exhibited disrupted ganglionic fusion (n=14). Four of these preparations also had successful intracellular cobalt fills of the FeDe MN, indicating that adult dendritic growth had been affected. For the oil-injected animals, FeDe muscles were dissected from 8 animals. Five of these preparations had successful intracellular cobalt fills of the FeDe MN indicating that normal adult dendritic growth had occurred. Leg muscles were stained as described above. For DAB processed muscles, 5 muscle fibers from one FeDe muscle were selected, and the nerve terminals within the 485 μm field of view were drawn using a *camera lucida* attached to a Zeiss compound microscope at 40x magnification. For fluorescently-labeled muscle fibers, a 3 dimensional image of the muscle fibers was collected using a confocal microscope at 40x magnification, which had a field of view of 280 μm. The total length of endplates on each muscle fiber was measured using NIH image software. The total length of nerve terminals on the 5 muscle fibers for a given preparation was averaged and then normalized to represent a 100 μm length of muscle fiber.

RESULTS

The central and peripheral remodeling of the FeDe MN during metamorphosis has been described previously (Kent and Levine, 1993; Knittel et al., 2001). In general, the growth of adult dendrites and motor terminals follows a similar time course, and both occur coincident with the pupal peak of ecdysteroids (Fig. 1B). The pupal peak of ecdysteroids is a single broad peak that occurs during *Manduca's* pupal stage (Fig. 1A; Bollenbacher et al., 1981). Interfering with the pupal ecdysteroid titer, either by eliminating the source of ecdysteroids (the prothoracic glands) or by treating with JHM, has been shown to prevent the growth of adult dendrites and motor terminals of respecified MNs (Truman and Reiss, 1988; Truman and Reiss, 1995). In my experiments, I used ligation to remove the source of the ecdysteroids to confirm that the pupal peak of ecdysteroids is required for adult differentiation of the FeDe MN's motor terminals. I subsequently used local JHM treatments of the CNS to determine the respective influence of central and peripheral ecdysteroid actions on the FeDe MN's adult dendritic growth and motor-terminal growth.

Ligation prevents adult differentiation of the FeDe MN's motor terminals

Ligated pupae were allowed to develop until matched unligated-control animals had become pharate adults. Examination of the metathoracic legs revealed that ligations on day P1 prevented the adult FeDe muscle from developing, and only a small muscle remnant that looked equivalent to a stage P1 FeDe muscle was in its place. Anti-synaptotagmin staining of the FeDe muscle remnant revealed a few scattered varicoscities associated with the remnant (n=3; arrows, Fig. 2A), which is characteristic of stage P1 FeDe muscle (Knittel et al., 2001). In contrast, anti-synaptotagmin staining

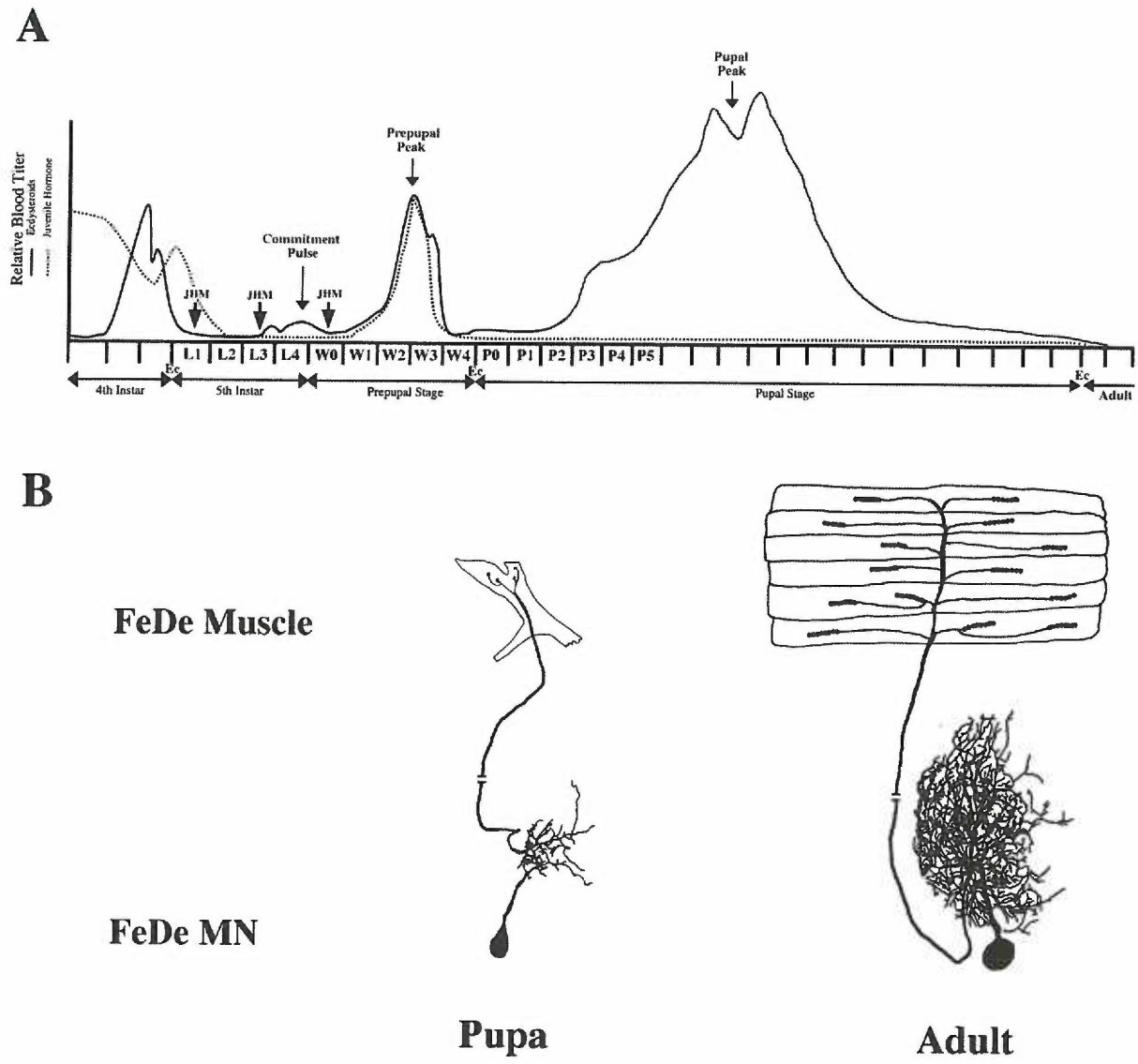

Figure 1. (A) Titers of ecdysteroids and JH during metamorphosis of Manduca sexta (adapted from Bollenbacher, et al., 1981). (B) Cartoon illustrating the changes that the FeDe MN and the FeDe muscle go through during adult development. Development of the adult FeDe muscle, elongation and differentiation of motor terminals, and growth of adult dendrites all occur during the pupal peak of ecdysteroids.

of adult FeDe muscles revealed many sprays of elongated boutons that covered the entire extent of the muscle (arrow, Fig. 2B). Elimination of the source of ecdysteroids by ligation, therefore, interfered with adult differentiation of the FeDe MN's motor terminals as well as the FeDe muscle. This is the first description of the effects of ligation on motor-terminal differentiation, and it complements previous work that demonstrated the effects of ligation on dendritic growth (Weeks and Ernst-Utzschneider, 1989). Like ligations, systemic injections of JHM have been shown to prevent adult differentiation of MNs if JHM is present during a sensitive period that spans the first 5 days of the pupal stage (Truman and Reiss, 1988; Truman and Reiss, 1995). Because both MN somata and muscles are known to be targets of the ecdysteroids (Hegstrom et al., 1998; Truman et al., 1994), ecdysteroids could be acting either centrally or peripherally to control adult differentiation of the FeDe MN's motor terminals.

Intraganglionic JHM injections on L3 prevents the elimination of larval dendritic processes and the growth of adult dendrites

In order to determine if motor-terminal growth and differentiation can occur independent of central ecdysteroid action, I used a heterochronic mosaic that had a preserved larval/pupal ganglion in an otherwise adult body. I injected JHM into the larval metathoracic ganglion (T3) on the third day of the fifth instar (L3) with the goal that the JHM would remain potent during the pupal peak of ecdysteroids. Control animals were injected with mineral oil to control for effects of the injection itself. This experimental manipulation led to normal development in the majority of the animals, although 4/42 control animals and 5/79 JHM-injected animals pupated incorrectly and were discarded from the study. After the completion of adult development, the FeDe MN

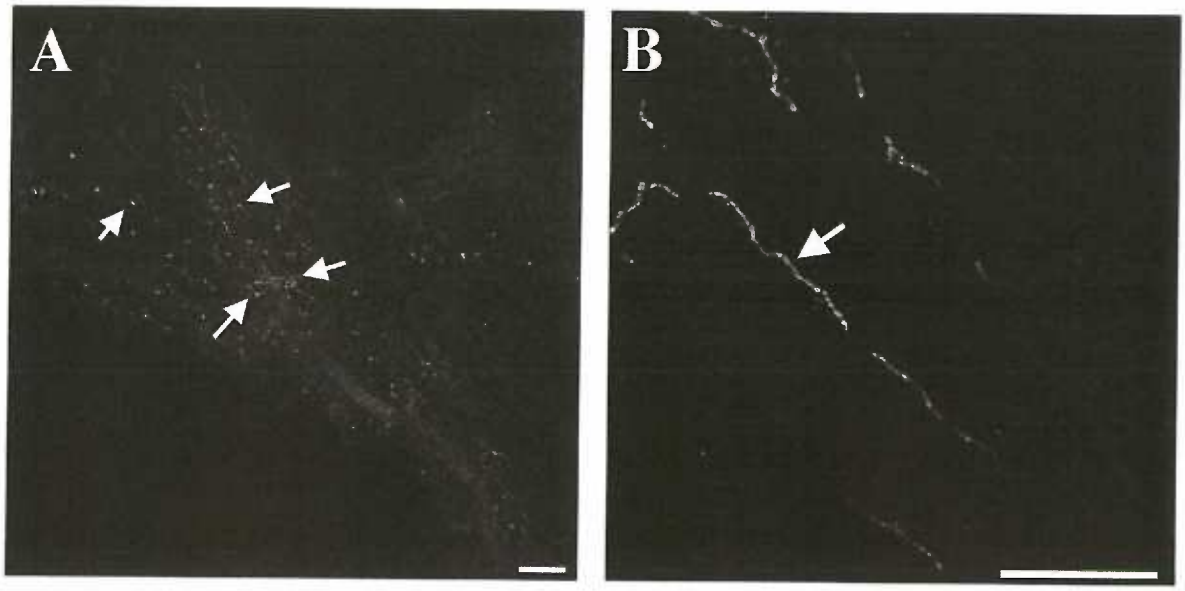

Figure 2. Ligation on day P1 prevents adult development of the FeDe MN's motor terminals and muscle. (A) FeDe muscle remnant from an animal ligated on day P1 and left for 18 days stained with Dsyt2. Only a few sparse varicoscities are present on the muscle remnant (arrows). (B) FeDe muscle from an adult stained with Dsyt2. Adult boutons form linear sprays along the muscle (arrow). Scale bar =100 μm in (A), and 50 μm in (B).

dendrites and motor terminals were examined from a subset of preparations in each group. Those preparations that did not display a retrogradely-labeled MN at the time of dissection were not included in the remainder of the study.

I examined the morphology of the FeDe MN's dendritic arbor in oil and JHM-injected animals using intracellular-cobalt staining. In 3/3 cobalt-stained oil-injected moths, the FeDe MN developed a normal adult dendritic arbor (Fig. 3A). In contrast, in 3/4 JHM-injected preparations, the FeDe MN did not develop a normal adult dendritic arbor (Fig. 3B). Of the 3 preparations that did not develop normal adult dendrites, 2 had a dendritic arbor with retained dorsal-medial processes that ended in enlarged varicoscities (arrow, Fig. 3B), which is a feature characteristic of regressing larval dendrites that is not seen in normal adults (Kent and Levine, 1993). One of the 4 JHM-injected preparations apparently was unaffected by the JHM and developed a dendritic arbor that was indistinguishable from oil-injected and normal adult preparations (data not shown). The 3 JHM-preparations that displayed abnormal adult dendritic growth were analyzed with a quantitative measurement of the FeDe MN's "dendritic extent", which revealed that the difference between oil- and JHM-injected animals was significant (Fig. 3C; $p=0.02$ one-tailed t test). The JHM treatment at L3, therefore, appeared to interfere with normal adult dendritic growth, and in some cases interfered with normal dendritic regression.

An unexpected effect of the JHM injection was that normal ganglionic fusion failed to occur, and this defect in fusion was correlated with disrupted dendritic morphology (compare Fig. 3A and 3B). Specifically, in all 3 cases in which dendritic morphology was affected, T3 failed to fuse with T2, although A1 and A2 fused with T3

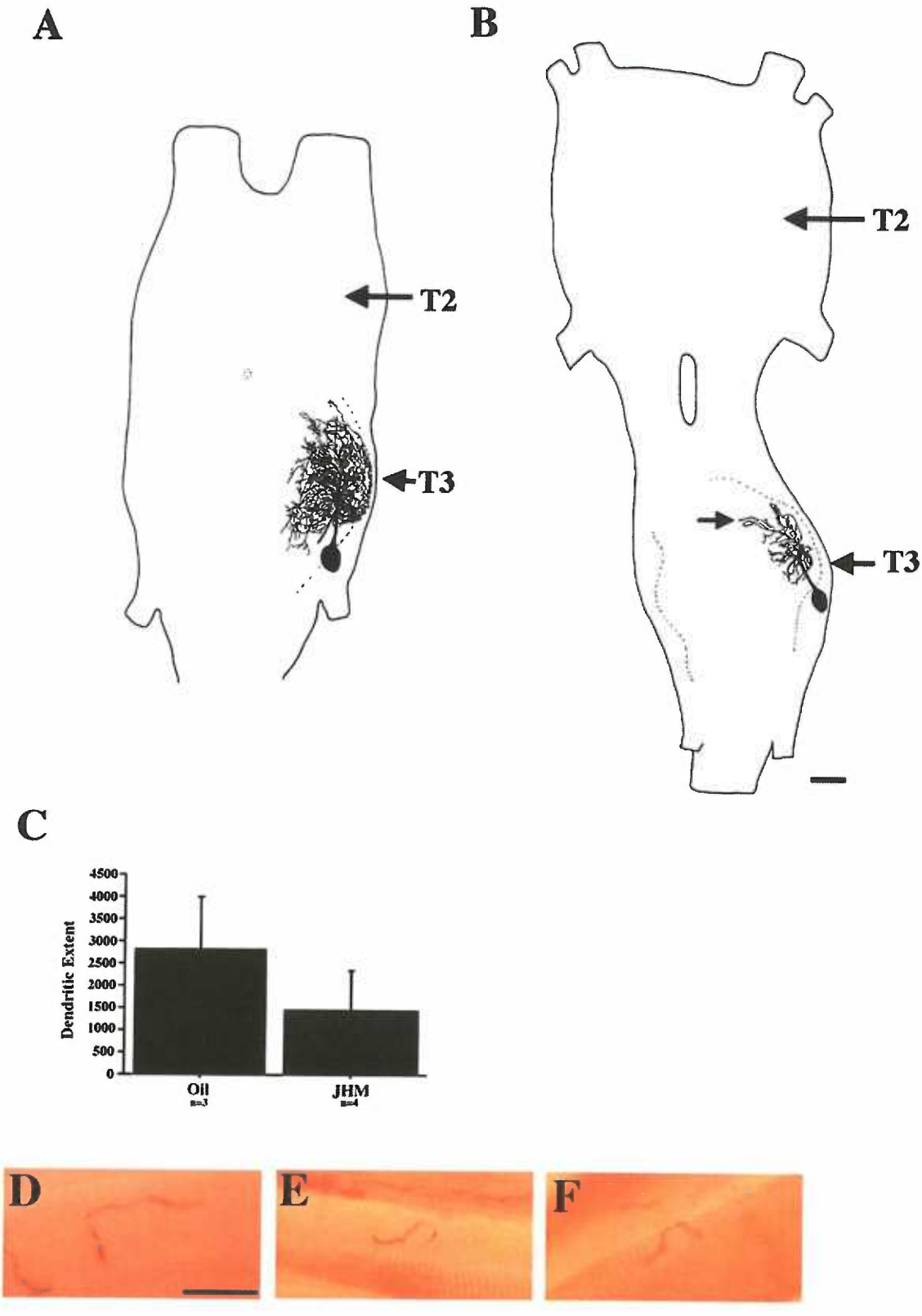
A
T2
T3
B
T2
T3
C
Dendritic Extent
4500
4000
3500
3000
2500
2000
1500
1000
500
0
OII
n=3
JHM
n=4
D
E
F

Figure 3

normally. The one JHM-injected preparation that exhibited normal adult dendrites also had normally fused ganglia (data not shown). All 3 of the oil-injected preparations exhibited normal ganglionic fusion. Therefore, the JHM treatment at L3 interfered with both dendritic growth and ganglionic fusion in the same preparations.

To determine if adult motor-terminal growth was affected by JHM treatment of T3, I dissected adult FeDe muscles from the animals in which I had successfully cobalt stained the FeDe MN. I labeled the FeDe muscles using anti-synaptotagmin antisera to reveal the morphology of the synaptic boutons. I found that in animals that had aberrant dendrites, the motor terminals on the adult FeDe muscles formed elongated sprays of boutons along all the muscle fibers (n=3; Fig. 3F). The appearance of the boutons was indistinguishable from oil-injected controls (n=3; Fig. 3E) and normal adults (Fig. 3D; Knittel et al., 2001). The JHM treatment, therefore, did not interfere with motor-terminal differentiation despite the effects on dendritic growth and ganglionic fusion. These results demonstrate that motor terminal differentiation can occur in the absence of the central effects of the ecdysteroids, and suggest that ecdysteroids act directly on peripheral targets to promote synapse formation.

I examined the morphology of the FeDe MN's dendrites at two stages during adult development to determine whether the dorsal-medial processes that appeared in 2/3 of the adult preparations were retained larval dendrites or re-grown aberrant processes. At stage P3, oil-injected preparations had undergone dendritic regression (n=2; Fig. 4A). The JHM-injected preparations, however, had many retained dorsal-medial processes that ended in enlarged varicoscities (n=8; arrows, Fig. 4B). At stage P7, one oil-injected preparation had begun dendritic growth and had formed a pterothoracic ganglion (n=1;

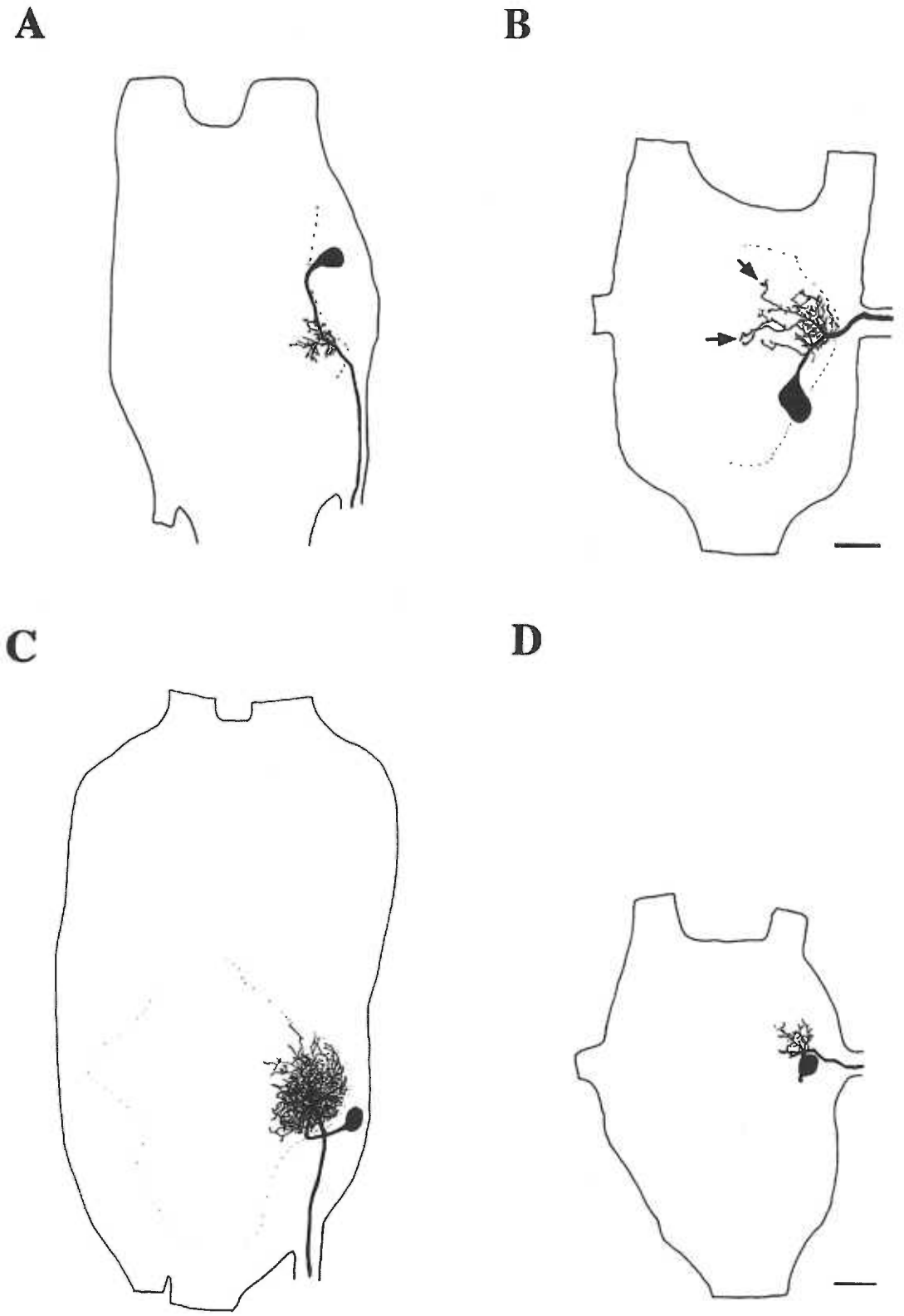

Figure 4. Camera lucida drawings of cobalt-stained FeDe MNs from animals that had either oil or JHM injected into T3 on L3. (A) At stage P3, an oil-injected animal has a FeDe MN that has undergone normal dendritic regression (B) At stage P3, a JHM-injected animal has retained larval dendritic processes (arrows). (C) At stage P9, an oil-injected animal has begun adult dendritic growth. (D) At stage P9, a JHM-injected animal appears to have undergone dendritic regression but has not yet begun dendritic growth. Scale bar = 100 μm for (A-D).

Fig. 4C). In contrast, at P7 one JHM-injected preparation had sparse dendrites with no dorsal-medial processes, which suggested that dendritic regression had occurred (n=1; Fig. 4D). Although the number of preparations examined was small, my results are consistent with the idea that the dorsal-medial processes seen in adult preparations represent retained larval dendrites. Together, these results demonstrate that JHM treatment at L3 interfered with dendritic growth as well as dendritic regression, suggesting that the treatment might have interfered with pupal commitment of the FeDe MN. To distinguish between the influence of JHM on pupal commitment and those on adult commitment, I treated animals with JHM at two additional time points.

Intraganglionic JHM injections on L1 prevents the elimination of larval dendritic processes and the growth of adult dendrites

I have shown previously that local JHM-injections into the larval ganglion prior to the commitment pulse of ecdysteroids prevents regression of larval dendrites (chapter 2; Knittel and Kent, 2002). To determine if those retained larval dendrites would persist during subsequent adult development, I examined similar preparations at the adult stage. Intraganglionic injections on the first day of the fifth instar (L1) led to normal development in the majority of the animals, although 3/30 control animals and 4/37 JHM-injected animals pupated incorrectly and were discarded from the study. After animals had completed adult development, the FeDe MN's dendrites and motor terminals were examined in a subset of preparations from each group. Only those with successful retrograde labels were processed further.

I found that the intraganglionic injection of JHM prior to the commitment pulse of ecdysteroids prevented the elimination of larval-like dendritic processes and affected the growth of adult dendrites. In 5/5 cobalt-stained oil-injected moths, the FeDe MN developed a normal adult dendritic arbor (Fig. 5A). In contrast, in 3/6 JHM-injected preparations, the FeDe MN did not develop normal adult dendrites (Fig. 5B). In 3 JHM-injected preparations, normal adult dendrites developed, and these preparations were not considered further. In the remaining 3 JHM-injected preparations, the FeDe MN's dendritic arbor had retained dorsal-medial processes that ended in enlarged varicoscities (arrows, Fig. 5B). Although the dendritic morphology appeared qualitatively different, measurements of the "dendritic extent" of the adult FeDe MN's arbor revealed that the difference was not statistically significant (Fig. 5C; $p=0.05$ one-tailed t-test). The lack of statistical difference between the oil and JHM-injected animals was probably due to the retention of larval dendrites. Examination of the FeDe muscles from JHM and oil-injected animals revealed that they formed normal adult synaptic endplates that were indistinguishable from normal adult endplates (data not shown). The JHM injection on day L1 also prevented formation of a pterothoracic ganglion in all 3 preparations that had aberrant adult dendrites (Fig. 5B). In contrast, the 3 JHM-injected animals that developed normal adult dendrites all exhibited normal ganglionic fusion (data not shown). In addition, all 5 oil-injected animals exhibited normal ganglionic fusion (Fig. 5A). Therefore, failure to undergo normal ganglionic fusion appears to be correlated with failure to develop normal adult dendrites.

I examined the morphology of the FeDe MN's dendrites at stage P3 and P9 to determine if the larval-like processes that are present in adult preparations represent

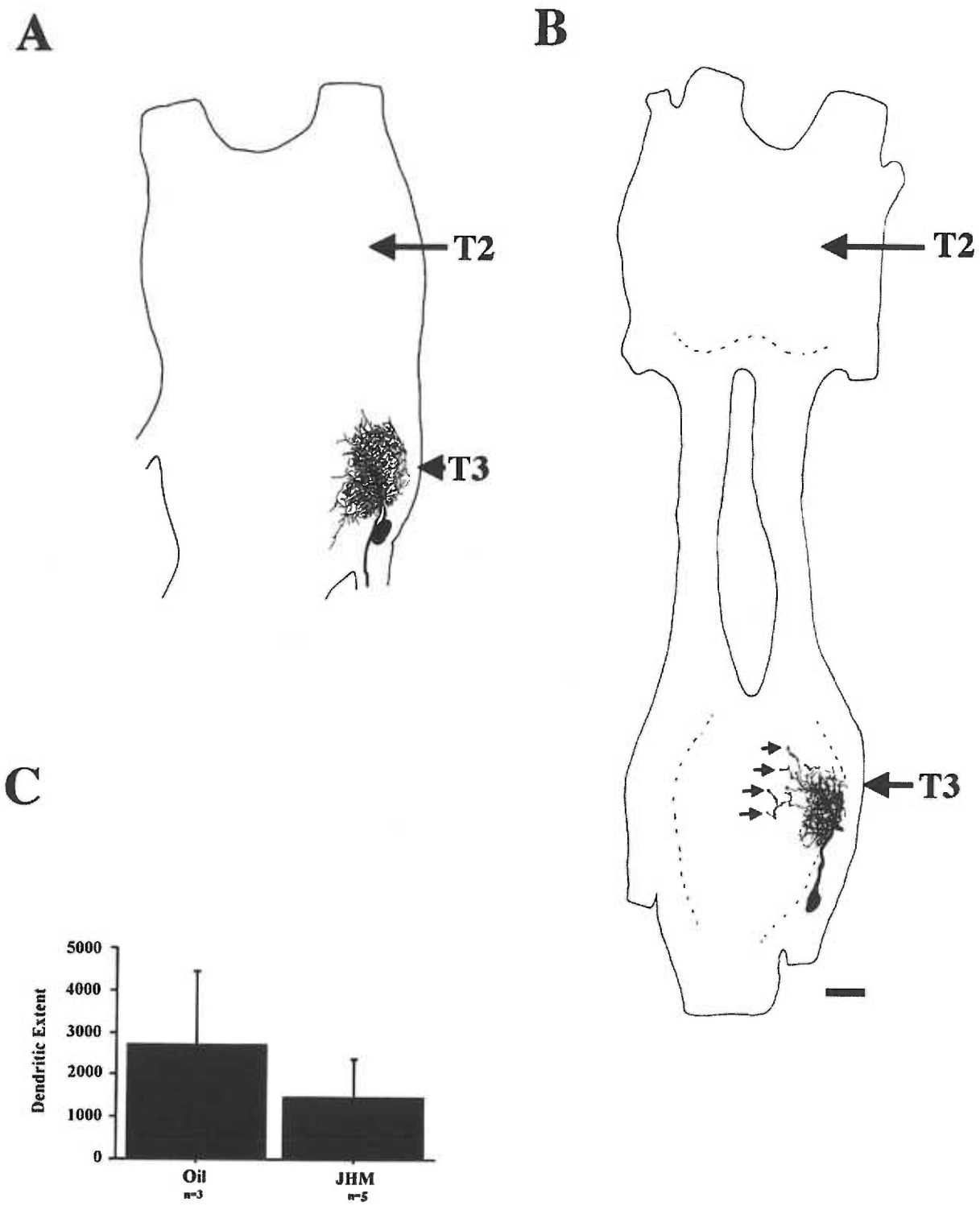

Figure 5. Camera lucida drawings of adult cobalt-stained FeDe MNs from animals injected with oil (A) or JHM (B) on the first day of the fifth instar (L1). (A) In the oil-injected animal, the FeDe MN develops a normal adult dendritic arbor. (B) In the JHM-injected animal, the dendritic arbor has many retained dorsal-medial processes (arrows). Fusion of T2 with T3 has not occurred. (C) Measurements of the "dendritic extent" of the FeDe MN from oil and JHM-injected ganglia reveal that the JHM-injected animals have fewer adult dendritic processes than the oil-injected animals. Average dendritic extent was 1448 pixels for JHM-injected animals and 2712 pixels for oil-injected animals. Scale bar = 100 μm for (A & B).

retained larval processes or re-grown processes. At stage P3, oil-injected preparations had undergone dendritic regression (n=4; Fig. 6A). The JHM-injected preparations, however, had a dendritic arbor with retained dorsal medial and lateral processes that resembled a larval dendritic arbor (n=8; Fig. 6B). At stage P9, oil-injected preparations had developed an extensive adult dendritic arbor and had formed a pterothoracic ganglion (n=2; Fig. 6C). In contrast, at P9 JHM-injected preparations still had retained lateral and dorsal-medial processes, but they appeared more sparse than at earlier stages, suggesting that some dendritic regression had occurred (n=3; Fig. 6D). Although new dendritic growth could not be ruled out, the FeDe MNs in these preparations appeared to be undergoing delayed dendritic regression rather than growth. Therefore, many of the processes seen in adult ganglia that had been JHM-injected prior to the commitment pulse (on day L1) appear to be retained larval processes.

Intraganglionic JHM injections on day W0 interferes with the growth of adult dendrites but does not affect the elongation of adult motor terminals.

To determine if JHM injections after the commitment pulse would affect the growth of adult dendrites without affecting dendritic regression, intraganglionic injections were made on day W0. Intraganglionic injections on day W0 led to normal development in the majority of the animals, although 7/29 control animals and 7/55 JHM-injected animals pupated incorrectly and were discarded from the study. After animals had completed adult development, the ganglia were dissected. Since previous experiments demonstrated a correlation between incomplete ganglionic fusion and aberrant dendritic morphology, I only analyzed the FeDe MN's dendrites and motor terminals from JHM-injected preparations that had disrupted ganglionic fusion.

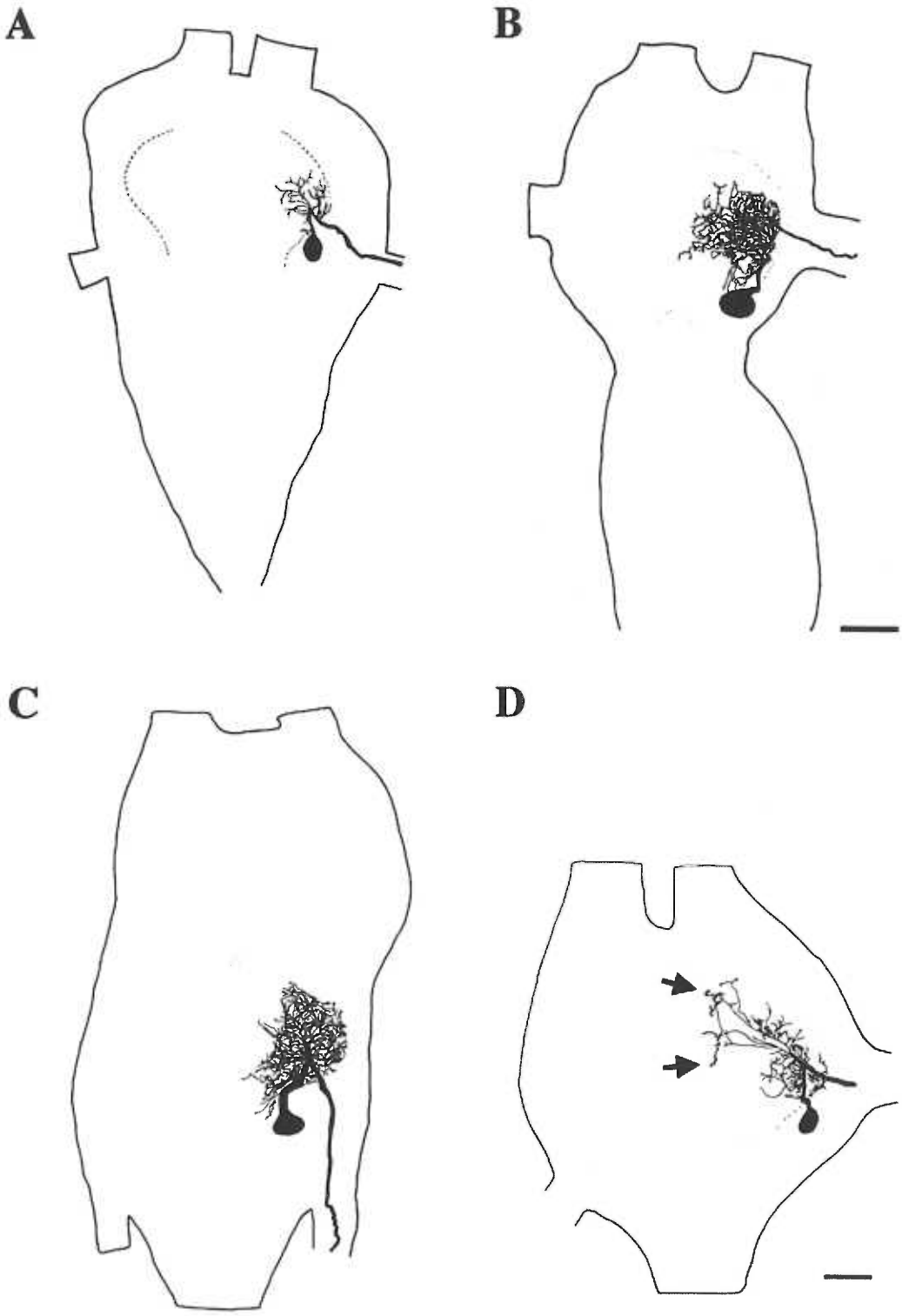

Figure 6. Camera lucida drawings of cobalt-stained FeDe MNs from animals that had either oil or JHM injected into T3 on L1. (A) At stage P3, an oil-injected animal has a FeDe MN that has undergone normal dendritic regression. (B) At stage P3, a JHM-injected animal appears to have a completely retained larval dendritic arbor, indicating that dendritic regression has not yet occurred. (C) At stage P7, an oil-injected animal has a FeDe MN that has begun adult dendritic growth. (D) At stage P7, a JHM-injected animal has a FeDe MN with many retained dorsal-medial processes (arrows) and some lateral processes, all of which probably represent retained larval dendrites. Scale bar = 100 μm for (A & B) and 100 μm for (C & D).

In 5/5 cobalt-stained oil-injected moths, the FeDe MN developed a normal adult dendritic arbor (Fig. 7A). In contrast, cobalt staining in 4/4 JHM-injected preparations with disrupted ganglionic fusion had FeDe MNs that did not develop normal adult dendrites (Fig. 7B). None of the JHM-injected preparations had dorsal-medial processes associated with the FeDe MN's dendritic arbor, which suggested that the injection interfered with adult dendritic growth without affecting dendritic regression. Measurements of the "dendritic extent" revealed that FeDe MNs from the JHM-injected animals had significantly fewer dendritic processes when compared to the oil-injected controls (Fig. 7C; $p=0.02$ one-tailed t-test). However, a small amount of dendritic growth did seem to have occurred. JHM treatment on day W0, therefore, interfered with both dendritic growth and ganglionic fusion.

Although ganglionic fusion and dendritic growth were altered in these preparations, the insect (including thoracic legs and leg muscles) developed normally. I examined the motor terminals on the FeDe muscles from these animals to determine if elongation and differentiation of adult synaptic boutons had occurred. I dissected FeDe muscles from JHM-treated animals that had disrupted dendritic growth and/or ganglionic fusion and found that the motor terminals had normal adult morphology (Fig. 8B & D). The endplates were simple elongated sprays characteristic of adult motor terminals (Fig. 2D; Consoulas et al., 1996; Knittel et al., 2001) and they were indistinguishable from the FeDe endplates from oil-injected control animals (Fig. 8A & C). The JHM injections did not appear to have any effect on the morphology of the adult FeDe muscles, which were the correct size and in the correct orientation. I counted the total length of endplates at the end of muscle fibers to determine if intraganglionic JHM injections affected

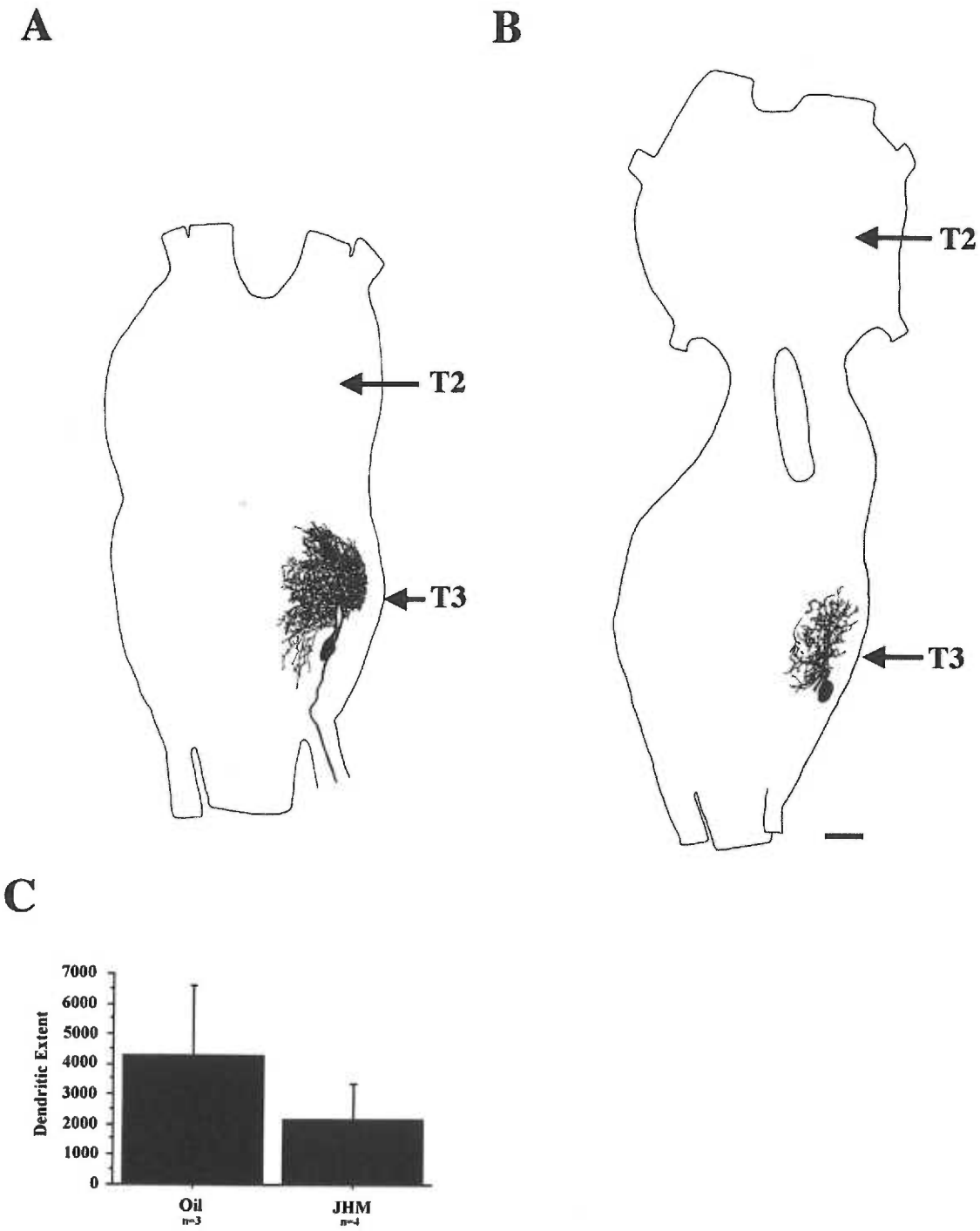

Figure 7. Camera lucida drawings of adult cobalt-stained FeDe MNs from animals injected with oil (A) or JHM (B) into T3 on day W0. (A) The oil-injected animal has a FeDe MN that developed a normal adult dendritic arbor. (B) The JHM-injected animal has a FeDe MN with stunted adult dendritic growth, and T2 failed to fuse with T3. The MN shown in (A) innervated the muscle shown in (Fig. 8 A & C), and the MN shown in (B) innervated the muscle shown in (Fig. 8 B & D). (C) Measurements of the "dendritic extent" of the FeDe MNs reveals that those from JHM-injected ganglia have fewer adult dendritic processes than those from oil-injected ganglia. Average dendritic extent was 2134 pixels for JHM-injected animals and 4256 pixels for oil-injected animals. Scale bar=100 μm in (A & B).

elongation of motor terminals. Quantitative analysis revealed no difference in the total length of endplates between JHM and oil-injected FeDe muscles (Fig. 8E). Thus, despite the influence of JHM on dendritic growth, the CNS injections had no apparent influence on motor-terminal elongation or differentiation. Normal muscle development was associated with normal motor terminals. These results suggest that interactions with the developing target muscle are sufficient to induce elongation and differentiation of adult motor terminals.

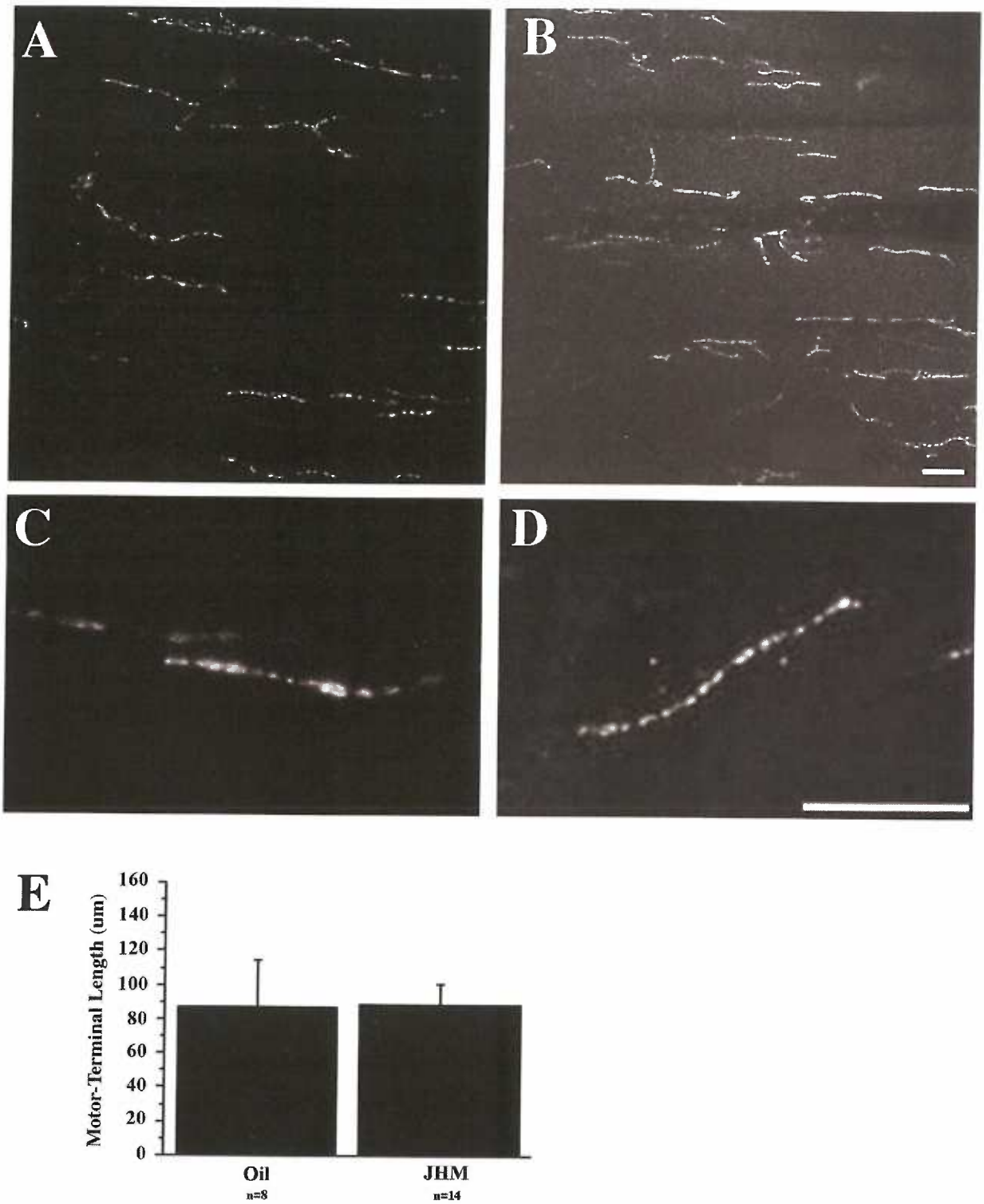

Figure 8. Dsyt2 stained adult FeDe muscles from animals that were injected with oil (A & C) or JHM (B & D) into T3 on day W0. The appearance of the adult FeDe MN endplates from JHM-injected animals is indistinguishable from oil-injected animals or normal adults. (E) Measurements of motor-terminal length from oil and JHM-injected animals reveals that intraganglionic injections of JHM have no effect on motor-terminal elongation. Average motor-terminal length/100 μm of muscle fiber was 87.4 μm for JHM-injected animals and 86.7 μm for oil-injected animals. Scale bar=100 μm in (A & B) and 25 μm in (C & D).

DISCUSSION

Remodeling of the FeDe MN during metamorphosis: role of the pupal peak of ecdysteroids

Growth of adult dendrites and elongation of FeDe motor terminals occur during the pupal peak of ecdysteroids (Kent and Levine, 1993; Knittel et al., 2001), suggesting that ecdysteroids control both events. In the current study, I confirmed that motor-terminal growth was prevented after the ecdysteroids were eliminated by ligating pupae on day P1 (Fig. 2A). Although I did not examine the MN's dendrites in ligated pupae, previous studies support the idea that 20E is responsible for adult dendritic growth. In studies on the VNL MNs, ligation at P1 prevented dendritic growth, and infusion of 20E into ligated abdomens promoted dendritic growth (Weeks and Ernst-Utzschneider, 1989). In addition, induction of pupae into diapause (a state of developmental arrest that prevents the pupal peak) prevents adult dendritic growth of abdominal MN-1, and infusion of 20E into diapausing pupae initiates the growth of MN-1's adult dendrites (Truman and Reiss, 1988).

The presence of JH at the beginning of the pupal peak of ecdysteroids has also been shown to block adult dendritic growth of MNs (Truman and Reiss, 1988). Experiments using systemic injections of JHM at the beginning of the pupal stage indicated that JHM can block adult dendritic growth and that each MN has a unique "JH-sensitive period" that occurs between stage P0 and P5. At the end of this sensitive period, JHM loses its ability to block adult-specific neurite outgrowth (Truman and Reiss, 1988). Although I did not determine the FeDe MN's JH-sensitive period, the local JHM injection clearly had an affect on adult dendritic growth (Figs. 3B, 5B & 7B). Ideally, my

intraganglionic injections of JHM should have been done on day P1 in order to interfere with adult commitment. However, because prepupae and new pupae were too fragile to undergo the surgery, I performed my injections on day W0. The influence on dendritic growth indicates that the JHM remained effective during the FeDe MN's JH sensitive period.

The presence of either systemic or peripheral JHM at the beginning of the pupal stage interferes with adult motor-terminal growth (Truman and Reiss, 1995). Systemic JHM injections have been shown to prevent elongation and differentiation of adult motor terminals (Truman and Reiss, 1995). In contrast, peripheral application of JHM to target muscle does not affect the elongation of motor terminals but does seem to affect the differentiation of motor endplates (Truman and Reiss, 1995). Those results suggested that ecdysteroid action on the MN soma was responsible for initiating the longitudinal growth of motor terminals, while the differentiation of endplates was due to local cell-cell interactions with the target muscle (Truman and Reiss, 1995). My results indicate that in the absence of central ecdysteroid action, interactions with the target muscle are sufficient for initiating elongation and differentiation of motor terminals. Local injections of JHM into ganglia on day W0 interfered with adult dendritic growth (Fig. 7B) without affecting the elongation or differentiation of adult motor terminals (Fig. 8B & E). In my experiments, the small amount of dendritic growth suggests that ecdysteroid action on the CNS might have been delayed rather than blocked entirely. If this were the case, delayed action of 20E on the MN soma might have allowed motor-terminal growth to progress more rapidly than dendritic growth, possibly because of synergistic effects of peripheral interactions. An examination of motor terminals at intermediate stages of

development would need to be done in order to address this possibility. Regardless of a potential delayed action of the ecdysteroids on the CNS, these results demonstrate that dendritic growth and motor-terminal growth are regulated differently. Peripheral influences of the ecdysteroids, therefore, are sufficient to promote normal motor-terminal growth but not dendritic growth.

Remodeling of the FeDe MN during metamorphosis: role of the commitment pulse of ecdysteroids

I have shown previously that the FeDe MN must experience the commitment pulse of ecdysteroids for larval dendritic regression and the final elimination of larval synaptic varicoscities to occur (Chapter 2; Knittel and Kent, 2002). Here, I show that the FeDe MN must experience the commitment pulse of ecdysteroids in order to undergo normal adult dendritic growth as well. Animals that received intraganglionic injections of JHM prior to the commitment pulse had adult FeDe MNs with stunted dendritic growth, retained larval processes, and unfused pterothoracic ganglia (Fig. 5B). MNs experienced delayed dendritic regression (Fig. 6B & D), but did not appear to undergo new adult dendritic growth. Therefore, exposing the MN to the pupal hormonal environment did not initiate the pupal pattern of development. Apparently, the MN first needs to go through the prepupal pattern of development (regression). Possibly, injection of JHM prior to the commitment pulse prevents the FeDe MN from expressing the ecdysone receptor isoform EcR-B1, which is responsible for directing the regressive effects of the ecdysteroids (Schubiger et al., 1998; Truman et al., 1994). Expression of EcR-B1 may have been induced later during the prepupal or pupal peak of ecdysteroids,

leading to the delayed regression of larval dendrites. Immunolocalization of ecdysone receptor isoforms would be needed to confirm this speculation.

Intraganglionic injections of JHM on L3 led to similar effects on adult dendritic growth as those seen in animals injected on L1. Adult dendritic growth was stunted, and larval dendritic processes were retained (Fig. 3B). The commitment pulse of ecdysteroids occurs on about the fourth day of the fifth instar (Bollenbacher et al., 1981). Therefore, intraganglionic injections of JHM on L3 probably blocked all or part of the commitment pulse. Injections on L3 did not have as severe an effect on dendritic regression when compared to injections made on L1, suggesting that the JHM was only partially effective at interfering with the commitment pulse.

Correlation between ganglionic fusion and dendritic remodeling

The pterothoracic ganglion is formed by the fusion of the first two abdominal ganglia (A1 & A2) with the last two thoracic ganglia (T2 & T3). During normal development, T3, A1, and A2 are fused together by stage P3, and by P7 fusion of T2 completes formation of the pterothoracic ganglion (Amos and Mesce, 1994). Normal ganglionic fusion has been shown to be controlled by 20E and only occurs in animals that have experienced all 3 pulses of ecdysteroids during metamorphosis (the commitment pulse, the prepupal peak, and the pupal peak; Amos et al., 1996). My results indicate that there is a correspondence between the failure to form a normal pterothoracic ganglion and the failure to execute normal adult dendritic growth. Within the group of animals injected with JHM on L1, 3 ganglia exhibited disrupted ganglionic fusion (Fig. 5D). All three of these ganglia had FeDe MNs with abnormal adult dendrites (Fig. 5B). In contrast, all 5 of the oil-injected ganglia exhibited normal ganglionic fusion and

developed normal adult dendrites (Fig. 5A). This correspondence between disrupted ganglionic fusion and disrupted dendritic growth allowed me to identify preparations in which the intraganglionic injections of JHM had been effective without having to label the FeDe MN intracellularly.

I believe that the intraganglionic injections of JHM had a local effect on the metathoracic ganglion, and that the effects on adult dendritic growth and ganglionic fusion do not represent systemic effects of the JHM. To rule out a systemic effect of JHM, I injected JHM into T1 on day L1. Ganglion T1 does not participate in the formation of the pterothoracic ganglion. If the JHM injection was affecting ganglionic fusion through a systemic effect, then I would expect the injection into T1 to inhibit ganglionic fusion. However, this preparation formed a normal pterothoracic ganglion, although the dendrites of the FeDe MN in T1 displayed stunted growth and retained dorsal-medial processes (data not shown). This suggested that the JHM injection into T1 had a local effect and only inhibited adult dendritic growth of the FeDe MN in that ganglion. In an additional preparation that had been injected with JHM into T3, I stained the FeDe MN in T2. Despite disrupted ganglionic fusion, the FeDe MN in T2 developed a normal adult dendritic arbor suggesting that the effects of JHM remained local and that events occurring in T3 are critical to normal ganglionic fusion (data not shown). The mechanisms responsible for ganglionic migration and fusion are unknown, but it has been speculated that the glial cells that make up the ganglionic sheath are involved (Amos and Mesce, 1994; Pipa, 1967). My results provide additional insight into the mechanisms underlying ganglionic fusion and suggest that local ecdysteroid-mediated events control fusion of T2 and T3. Perhaps JHM had a local effect on the glial cells that ensheath T3,

which in turn was responsible for the failure of T3 to fuse with T2. Alternatively, JHM treatment of T3 might have interfered with ecdysteroid-mediated shortening of axons belonging to interganglionic interneurons with cell bodies located in T3. Although I did not inject JHM into T2, a similar effect on ganglionic fusion might have occurred.

Mechanisms of peripheral influences on MN terminal growth

During remodeling of the neuromuscular system, growth of new adult muscles occurs in close association with motor terminals. The muscle is a direct target of the ecdysteroids, and ecdysteroid exposure as well as contact with the nerve is required for adult muscle development (Hegstrom et al., 1998; Luedeman, 1996). During motor-terminal remodeling, motor terminals retract away from degenerating larval muscles (Knittel and Kent, 2002; Truman and Reiss, 1995) but retain contact with the anlage of the adult muscle (Consoulas et al., 1996; Knittel et al., 2001). The nerve appears to influence myoblast proliferation and accumulation (Bayline et al., 2001; Consoulas and Levine, 1997; Luedeman, 1996), while the muscle appears to influence motor-terminal branching, the rate of motor-terminal growth, and endplate differentiation (Bayline et al., 2001; Fernandes, 1998; Truman and Reiss, 1995). My experiments support previous suggestions that target muscle influences endplate differentiation and further suggests that interactions with the target muscle are sufficient for both motor-terminal differentiation and elongation.

Several experiments have supported the idea that motor-terminal growth could be locally controlled. Studies on the developing dorsolongitudinal flight muscles (DLMs) in *Manduca* have indicated that the rate of motor-terminal growth is regulated by the size of the target muscle (Bayline et al., 2001). In these experiments, denervation of DLMs 1-4

led to the development of a hypertrophied DLM5 anlage, and the growth rate of motor terminals over this larger muscle was faster when compared to control muscles (Bayline et al., 2001). Laser ablation studies on the DLMs in *Drosophila* showed that development of MN secondary branches on the target muscle was dependent upon interactions with the muscle (Fernandes, 1998).

Specific molecules that control motor-terminal growth have been identified. Mutations in the *Drosophila* gene *kakapo* cause reduced motor-terminal branching, and reduced size and number of synaptic boutons (Prokop et al., 1998). This effect is not due to a global defect in axonal growth because MNs are able to reach their target muscle (Prokop et al., 1998). Additionally, mutations in *kakapo* cause an inappropriate upregulation of Fas II in neuronal processes (Prokop et al., 1998). Fas II expression at the NMJ has been shown to be required for growth of the synapse in *Drosophila*, and altering levels of expression of Fas II at the synapse regulates the amount of growth (Schuster, 1996a). Levels of Fas II are thought to control the structural aspects of synapse growth in a signal transduction cascade that is downstream of synaptic activity and cAMP levels (Schuster et al., 1996b). The Fas II model provides insight into how motor-terminal growth might be occurring in the absence of ecdysteroid action on the FeDe MN cell body. Regulation of Fas II expression at the FeDe NMJ by peripheral ecdysteroid action could serve to direct motor-terminal growth in the absence of ecdysteroid action on the MN cell body. Further studies on manipulating levels of Fas II *in vivo* would be needed to confirm this hypothesis.

The ecdysteroids could possibly have other peripheral actions besides acting directly on the target muscle. Perhaps peripheral glia are direct targets of the

ecdysteroids. Our previous studies have shown that motor-terminal growth is concurrent with the growth of peripheral glial processes that ensheath the motor nerve (Knittel et al., 2001). Ecdysteroids could potentially act directly on glia to trigger their migration, which in turn causes the MN processes to follow. Alternatively, the ecdysteroids could be having a nongenomic action on the motor terminals by acting on membrane receptors to influence intracellular calcium levels, which could in turn lead to neurite outgrowth. Although both of these ideas are a possibility, there is no evidence to support this role for the ecdysteroids.

Mechanisms of central influences on dendritic growth

The evidence to date suggests that ecdysteroids initiate growth of central neuronal processes via a direct action on the cell body. Local manipulations of retained larval sensory neurons indicated that the hormonal environment of the cell body determines whether or not the neuron will sprout pupal-specific processes (Levine, 1986). Later, studies on cultured leg MNs indicated that 20E enhances the complexity of process branching (Matheson and Levine, 1999; Prugh, 1992). However, the best evidence that ecdysteroids act directly on the cell bodies of neurons comes from autoradiographic studies that showed that central neurons accumulate ponasterone A, an ecdysone agonist (Fahrbach, 1992; Fahrbach, 1989). In addition, immunolocalization indicates that MNs express two isoforms of the ecdysone receptor (EcR) and that expression of the EcR-B1 isoform is associated with regressive aspects of remodeling, while expression of the EcR-A isoform is associated with growth (Truman et al., 1994).

The presence of JHM during the early pupal stage can block ecdysteroid-mediated dendritic growth (Figs. 3B, 5B, & 7B; Truman and Reiss, 1988), but the mechanisms by

which JHM does this are not well understood. JH modulates the actions of the ecdysteroids by preventing the ecdysone-induced switching in gene expression required for metamorphosis (Riddiford, 1996a). Exposure of larval cuticle to JH during pupal commitment prevents the expression of the Broad-Complex gene, an ecdysone-inducible gene that is one of the first expressed at the onset of metamorphosis (Zhou et al., 1998; Zhou and Riddiford, 2001). Similarly, JH could prevent adult commitment by preventing MNs from responding to 20E and initiating the expression of adult-specific genes that are required for adult dendritic growth. Presumably, these genes would include genes for cytoskeletal proteins or proteins that influence cytoskeletal assembly or stability.

Besides direct actions of ecdysteroids on MNs, indirect actions could be playing a role in triggering dendritic growth. Afferent input has been implicated in controlling the shape of the adult dendritic arbor of the FeDe MN (Kent and Levine, 1993), but my experiments indicate that afferent input is not sufficient for normal adult dendritic growth. Target muscle ablation has been correlated with precocious dendritic growth of MN5 in *Drosophila* (Fernandes, 1998), but target muscle development did not seem to promote dendritic growth in my experiments. The absence of central glial cells in a *Drosophila* mutant has been shown to affect dendritic growth (Hosoya et al., 1995; Jones et al., 1995), and in my experiments the glial cells and other central neurons were also exposed to the JHM treatment. Therefore, in my experiments the lack of ecdysteroid effects on other central cells might have contributed to the effects that I observed on dendritic growth.

Uncoupling of dendritic growth and motor-terminal growth

I have shown previously that the FeDe MN's dendritic regression and motor-terminal retraction can be experimentally uncoupled, suggesting that they are regulated by different mechanisms (chapter 2; Knittel and Kent, 2002). Moreover, specific aspects of motor-terminal retraction can also be uncoupled (Knittel and Kent, 2002). Here, I show that the FeDe MN's dendritic growth and motor-terminal growth can also occur independent of each other. As was the case for the regressive aspects of FeDe MN remodeling, growth of processes at opposite ends of the MN seems to be regulated differently. The picture emerging from several studies indicates that the ecdysteroids do not simply initiate a uniform regressive or constructive response by triggering a stereotypical pattern of gene expression in the MN. Rather, I have found that adult dendritic growth can be experimentally uncoupled from adult motor-terminal growth, suggesting that each process is regulated by separable mechanisms. In addition, I found that peripheral ecdysteroid action is sufficient to promote both elongation and differentiation of motor terminals. My results, together with those of Truman & Reiss (1995) suggest that either central or peripheral ecdysteroid actions may be sufficient to induce the growth and elongation of motor axons and branches.

CHAPTER 4

Regulation of TM-MFasciclin II Expression by the Femoral Depressor Motoneuron During Metamorphosis

SUMMARY

During metamorphosis of the tobacco hawkmoth *Manduca sexta*, the femoral depressor motoneuron (FeDe MN), undergoes remodeling of its central and peripheral processes. Regression of larval dendrites and retraction of larval motor terminals is followed by the growth of adult dendrites and the elongation of adult motor terminals. The regressive aspects of FeDe MN remodeling are controlled by central and peripheral actions of the ecdysteroids, the steroid hormones that control insect metamorphosis. Little is known about ecdysteroid-regulated molecules, or how they may be involved in MN remodeling. We have shown previously that the FeDe MN begins to stain with antisera against the transmembrane isoform of *Manduca* fasciclin II (TM-MFas II) for the first time during the regressive phase of MN remodeling, suggesting that it may be regulated by the prepupal peak of ecdysteroids. In this study, I have asked whether the appearance of TM-MFas II immunoreactivity in the FeDe MN soma is controlled by the ecdysteroids, acting either centrally or peripherally. My results indicate that TM-MFas II immunoreactivity in the FeDe MN is increased by at least two mechanisms: central ecdysteroid action and peripheral axotomy.

INTRODUCTION

Holometabolous insects go through a hormonally-regulated remodeling of their neuromuscular system during metamorphosis. Larval muscles that are not needed for pupal and adult behaviors die, and some of the motoneurons (MNs) that innervated these muscles are respecified to innervate new adult target muscles. The central and peripheral processes of these respecified MNs are remodeled in order to accommodate changes in synaptic connectivity. Larval dendrites and motor terminals regress before being replaced by new adult dendrites and motor terminals. The insect steroid hormone 20-hydroxyecdysone (20E) has been shown to control both central and peripheral remodeling of respecified MNs (Knittel and Kent, 2002; Truman and Reiss, 1988; Truman and Reiss, 1995; Weeks and Ernst-Utzschneider, 1989; Weeks and Truman, 1985).

Insect metamorphosis is controlled by the ecdysteroids (primarily by 20E) and juvenile hormone (JH). During the fifth and final instar, a small rise in the ecdysteroid titer occurs in the absence of JH for the first time (Bollenbacher et al., 1981). This "commitment pulse" of ecdysteroids is responsible for reprogramming larval tissues to a pupal fate (reviewed by (Riddiford, 1996b). The subsequent "prepupal peak" of ecdysteroids occurs in the presence of JH and initiates the pupal molt. A final sustained rise in the ecdysteroid titer called the pupal peak occurs in the absence of JH and directs adult development.

Insect muscle and MNs are both targets of ecdysteroid action, and central and peripheral ecdysteroid action is responsible for distinct aspects of MN remodeling. Regression of larval dendrites is controlled by central ecdysteroid action (Knittel and

Kent, 2002; Weeks and Truman, 1986), while retraction of larval motor terminals is controlled by peripheral ecdysteroid action (Knittel and Kent, 2002; Truman and Reiss, 1995). Ecdysteroid action on peripheral tissues mediates motor-terminal retraction, possibly via local cell-cell interactions (Knittel and Kent, 2002; Hegstrom and Truman, 1996b). Peripheral ecdysteroid actions include degeneration of larval target muscle, which suggests that nerve-muscle interactions might control retraction of larval motor terminals. The molecules that mediate cell-cell interactions during hormonally-mediated remodeling have not been identified.

Cell adhesion molecules (CAMs) play important roles in mediating neurite outgrowth and in organizing the structure of the NMJ (reviewed by Doherty et al., 1995; Goodman, 1996; Sanes and Lichtman, 1999). In vertebrates, the neural cell adhesion molecule (NCAM) is expressed by motor axons during development or during reinnervation but becomes restricted to the NMJ once a synapse differentiates (Covault and Sanes, 1985; Covault and Sanes, 1986). In the mollusk *Aplysia*, synaptic growth is preceded by a downregulation of apCAM, a homologue of NCAM. Another invertebrate homologue of NCAM is fasciclin II (Fas II), which has been shown to control synaptic stabilization and growth in *Drosophila* through regulation of its expression level (Schuster, 1996a; Schuster et al., 1996b). A transmembrane (TM) and a GPI-linked isoform of Fas II have been cloned from *Manduca* (MFas II; Wright et al., 1999), and we have previously described the expression pattern of both isoforms at the FeDe NMJ during metamorphic remodeling (Knittel et al., 2001). We found that TM-MFas II immunoreactivity (TM-MFas II-IR) was detectable on the somata and processes of the FeDe MN only during prepupal and early pupal stages. In contrast, GPI-MFas II-IR was

detectable on glial cells that ensheath the axons and processes of motor and modulatory neurons throughout postembryonic life (Knittel et al., 2001).

The apparent increase in TM-MFas II expression by the FeDe MN was coincident with both the prepupal peak of ecdysteroids and degeneration of the larval FeDe muscle, suggesting that either central or peripheral ecdysteroid actions could regulate TM-MFas II. Upregulation of TM-MFas II could, in turn, play a role in cell-cell interactions during remodeling of the FeDe MN's motor terminals. Here, as a first step towards understanding the peripheral cues involved in motor-terminal remodeling, I have investigated if expression of TM-MFas II is regulated by the ecdysteroids or by peripheral interactions. A preliminary account of these results has been presented in abstract form (Knittel et al., 1999).

MATERIALS AND METHODS

Animals

Manduca sexta (Lepidoptera: Sphingidae) were obtained from a laboratory culture reared on an artificial diet (Bell and Joachim, 1976) and a long-day photoperiod regimen (17:7 h light/dark) at 26 ^{o}C and approximately 60% relative humidity. Developmental stages were monitored by both chronological and morphological criteria (Nijhout and Williams, 1974; Reinecke et al., 1980; Tolbert et al., 1983; Truman and Riddiford, 1974; Truman et al., 1980). W0 signifies the first day of "wandering," and W1, W2, etc., refer to the ensuing prepupal days. W0 stage larvae were identified by the exposure of the dorsal vessel, which indicates that they had experienced the commitment pulse of ecdysteroids (Truman and Riddiford, 1974). P1 signifies the first day after the molt to the pupal stage. Prior to surgical or experimental manipulation, animals were anesthetized either by immersion in ice water or exposure to CO_2.

Hormonal manipulations

To eliminate the source of ecdysteroids, larvae were anesthetized on the first day of the 5th instar (L1), at 8:00 p.m. on the evening of the fourth day of the 5th instar (L4 p.m.), or at 10:00 a.m. on the morning of the first day of the wandering stage (W0 a.m.). Animals were ligated with silk suture through the middle of the mesothoracic segment. The head and prothoracic segment were excised and discarded. Four days after ligation, the metathoracic ganglion was dissected and fixed for immunohistochemistry.

To distinguish between peripheral and central influences of ecdysteroids on MFas II expression, we used heterochronic mosaic insects in which peripheral tissue and the metathoracic ganglion were at different developmental stages, as described previously

(Knittel and Kent, 2002). One larval ganglion was arrested in the larval stage while the remainder of the animal underwent pupal development. The metathoracic ganglion was injected with a juvenile hormone mimic (JHM; S-methoprene; gift of Sandoz, Inc.; 1μg/ml in mineral oil) on the first day of the fifth instar to prevent pupal commitment of the FeDe MN. Metathoracic ganglia were surgically exposed, immobilized on a platform, and pressure-injected with 0.1-0.3 μl of JHM or mineral oil using a PicoPump (WPI, Inc.). A few crystals of PTU (phenlythiourea; Sigma) were inserted into the wound to prevent oxidation of the hemolymph. The incision was sutured closed and sealed with Vetbond (3M). On day P1, metathoracic ganglia were dissected and fixed for immunohistochemistry.

Intracellular labeling of MNs

To visualize the cell body of the FeDe MN in the CNS, a non-toxic, persistent fluorescent dye (Rhodamine Dextran Lysine 3000 MW or Alexa 488 Dextran Lysine 10,000 MW, 5% in 2% KCl; Molecular Probes) was pressure injected into the region of the FeDe muscle to retrogradely label the MN soma (Kent and Levine, 1993; Knittel et al., 2001). Dye injections were made either during the 4th larval instar or on the first day of the 5th instar.

Immunocytochemistry

To label the cells in the metathoracic ganglion that express the TM isoform of MFas II, dissections were performed in PBS, and the tissue was fixed in Bouin's solution + 0.1% TX at 4° C overnight. The tissue was subsequently rinsed for 24-48 hours in 70% ethanol saturated with lithium carbonate to remove the picric acid. After being returned

to PBS through a graduated ethanol series, tissue was incubated in 10% normal goat serum (NGS) for 1-24 hr. Anti-TM-MFas II antisera (anti-TM-MFas II) were used at 1:2000 in PBS + 0.2% TX and 5% NGS for 24-72 hours. The tissue was processed for immunohistochemistry using an Alexa-Fluor 488 or 546-conjugated goat-anti-guinea pig antibody (Molecular Probes).

Axotomy

On day 1 of the 5th instar (L1), larvae were immobilized and an incision was made along the ventral midline to expose the metathoracic ganglion. The right ventral nerve or both anterior connectives were severed. A few crystals of PTU were inserted into the wound, and the wound was sutured closed and sealed with Vetbond (3M). Four days after axotomy, the metathoracic ganglion was dissected, fixed in Bouin's, and prepared for immunohistochemistry.

Image Processing

Image processing consisted of adjusting contrast and brightness of the images using Adobe Photoshop 4.0 software. For the intraganglionic injection experiments, the green FeDe MN label was reassigned as red for consistency of comparison with previous figures.

RESULTS

TM-MFas II-immunoreactivity in the FeDe MN is first detectable during the prepupal stage

We have shown previously that TM-MFas II becomes detectable immunohistochemically in the FeDe MN's soma on day W2, coincident with rising titers of the prepupal ecdysteroids (Knittel et al., 2001). To confirm that the FeDe MN does not label with TM-MFas II antisera prior to the wandering stage, I examined TM-MFas II immunoreactivity (IR) in FeDe MNs on the fourth day of the 5th instar (L4) and on the first day of the wandering stage (W0). I found that TM-MFas II-IR in the FeDe MNs was not detectable in stage L4 larvae (Fig. 1A-C) or stage W0 larvae (Fig. 1D-F). In fact, very few central neurons express TM-MFas II during the 5th instar. A few small somata in the anterior packets of cells are labeled (Fig. 1B), as are the ventral unpaired median (VUM) neurons (not visible in optical sections through the dorsal region of the ganglion; Knittel et al., 2001). By contrast, the FeDe MNs at later prepupal stages (W2-W4) have detectable TM-MFas II-IR (Fig. 1G-I), as do many additional central somata (Knittel et al., 2001).

The appearance of TM-MFas II-IR in the FeDe MN is prevented by ligation on L4 p.m.

To determine if the prepupal peak of ecdysteroids upregulated expression of TM-MFas II in the FeDe MN during the prepupal stage, I ligated animals on the morning of day W0 (W0 a.m.) and then examined the FeDe MNs four days later. Surprisingly, ligating at this time did not prevent the appearance of TM-MFas II-IR in the FeDe MNs (arrows, Fig. 2A-C). To test if the commitment pulse of ecdysteroids was the signal responsible for the apparent upregulation of TM-MFas II, I ligated animals on day 1 of

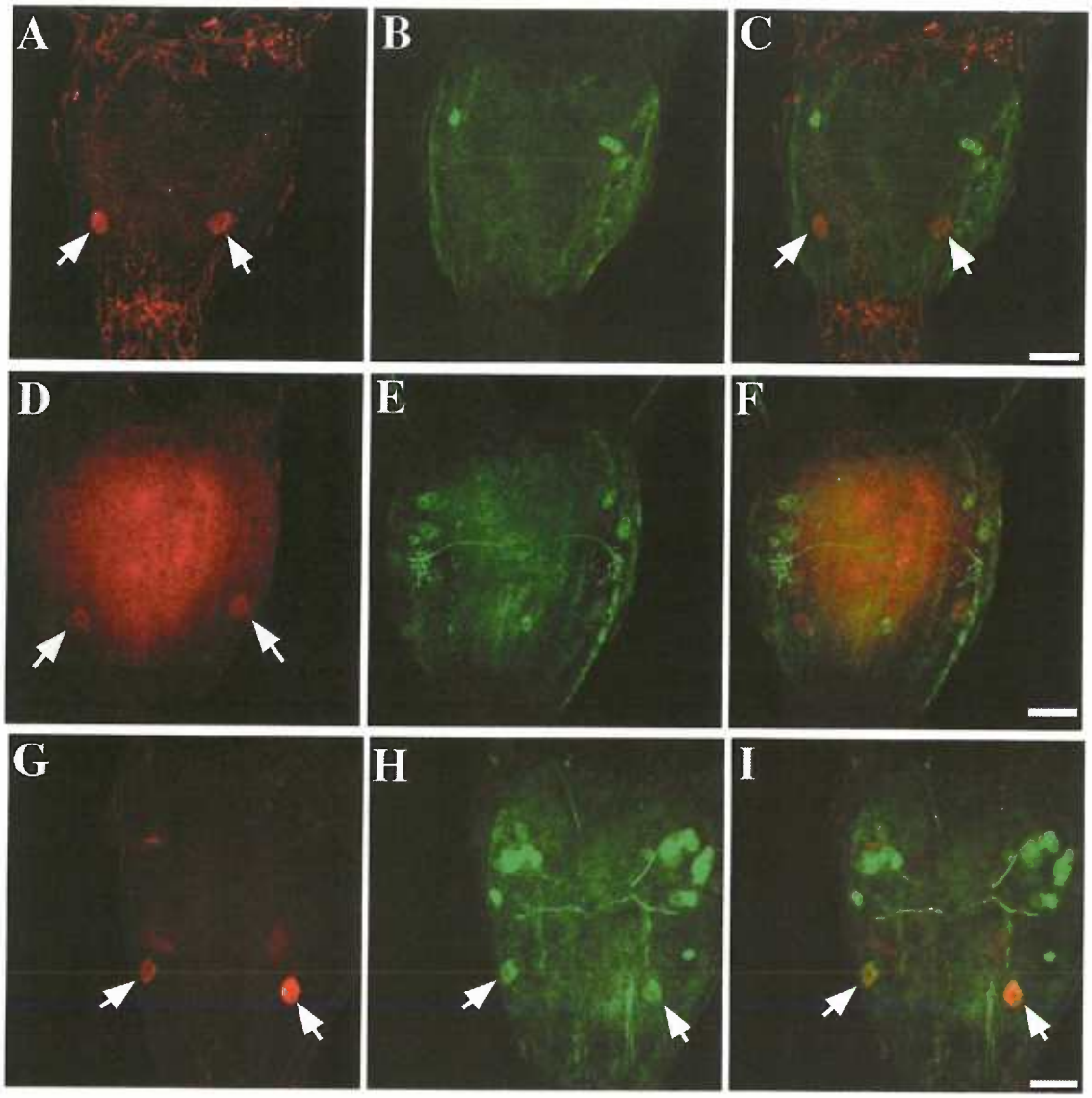

Figure 1. Expression of TM-MFas II by the FeDe MN during normal development. (A-C) Fourth day of the fifth instar (L4). (D-F) First day of wandering stage (W0). (G-I) Fourth day of wandering stage (W4). (A) RDL backfill of the FeDe MNs (arrows) from a L4 larva. (B) TM-MFas II staining labels very few neurons and processes at L4. (C) Composite of A & B indicating that the FeDe MNs do not express TM-MFas II at L4. (D) RDL backfill of the FeDe MNs (arrows) from a stage W0 larva. (E) TM-MFas II staining labels very few neurons and processes at stage W0. (F) Composite of D & E indicating that the FeDe MNs do not express TM-MFas II at stage W0. (G) RDL backfill of the FeDe MNs (arrows) from a W4 prepupa. (H) TM-MFas II staining labels the FeDe MNs (arrows) and many other neurons and processes at stage W4. (I) Composite of G & H indicating that the FeDe MNs express TM-MFas II at stage W4. Scale bar in C = 100 μm (same magnification as A & B), 100 μm in F (same magnification as D & E), and 100 μm in I (same magnification as G & H).

the 5th instar (L1), which should prevent the commitment pulse and the prepupal peak. When I examined those preparations 4 days later, I found that ligating prior to the commitment pulse prevented the appearance of TM-MFas II-IR in the FeDe MNs (Fig. 2E-G), indicating that the commitment pulse alone could be responsible for the labeling seen in animals ligated on W0 a.m. In addition, this manipulation indicated that the surgical procedure of ligation was not responsible for upregulating TM-MFas II expression. To investigate further the role of the commitment pulse, I ligated animals on the evening of the fourth day of the 5th instar (L4 p.m.). In the morning, these animals had exposed their dorsal vessel, indicating that they had experienced the commitment pulse (Truman and Riddiford, 1974) despite the earlier time of ligation. When I examined the FeDe MNs from these animals 4 days later, I found that the appearance of TM-MFas II-IR had been prevented (Fig. 2G-I). This result suggests that despite the apparent commitment of the body tissues, the FeDe MN was not fully committed on L4 p.m. and that ligations might have shortened the commitment pulse slightly. Thus, expression of TM-MFas II could depend upon full exposure to the commitment pulse while other aspects of commitment require a shorter exposure.

Axotomy leads to an increase in TM-MFas II immunoreactivity in the ipsilateral FeDe MN

The developmental appearance of TM-MFas II-IR could have been due to ecdysteroid action on the FeDe MN cell body or on the larval FeDe muscle. Ecdysteroids act directly on the FeDe muscle to initiate its degeneration, and a change in interactions with the target muscle could, in turn, influence expression of TM-MFas II in the FeDe MN (Knittel and Kent, 2002). To address the possibility that a change in interactions

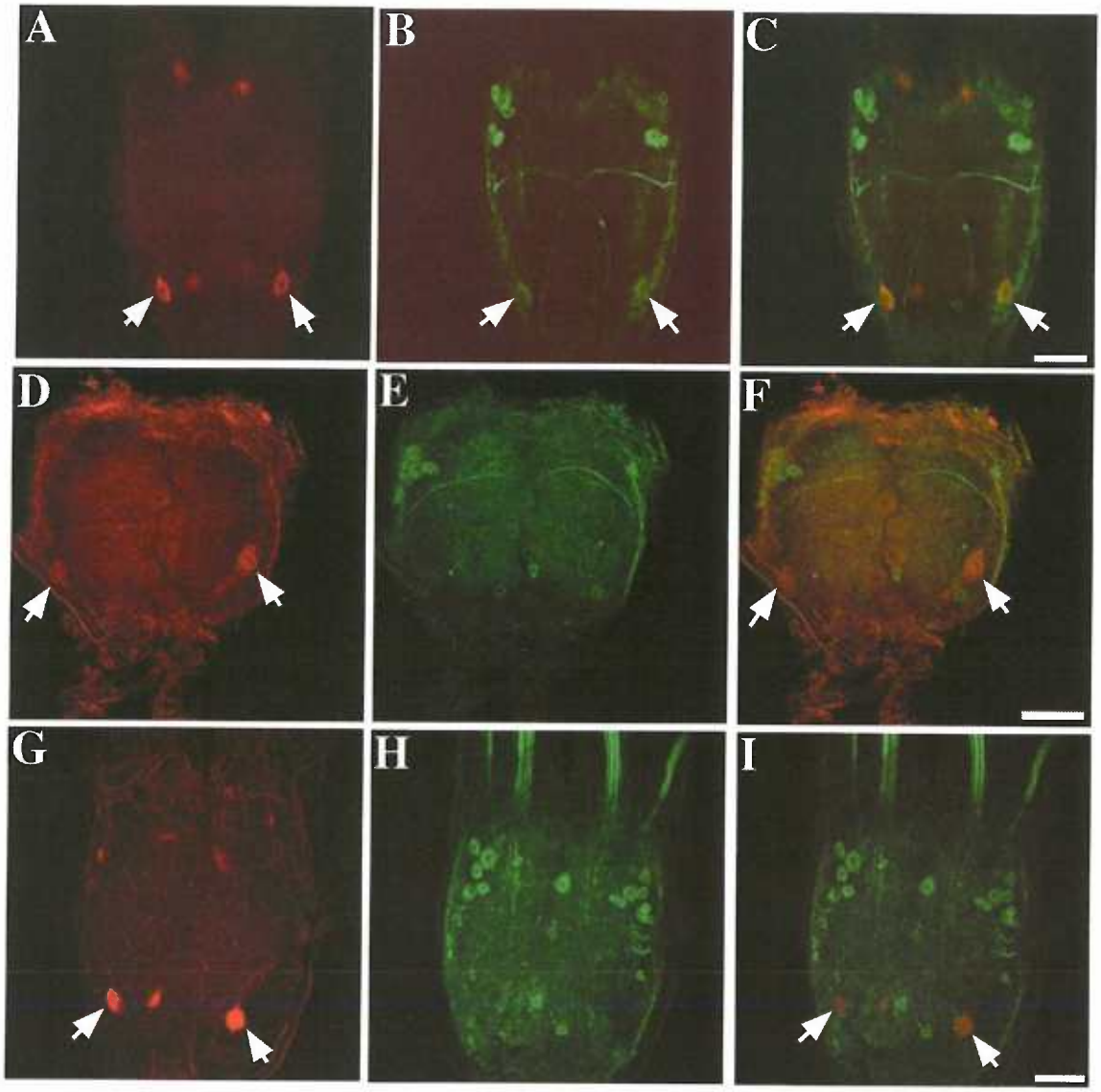

Figure 2. Expression of TM-MFas II by the FeDe MN in ligated animals. (A-C) Ligated on W0 a.m., (D-F) ligated on L1, and (G-I) ligated on L4 p.m. (A) RDL backfill of the FeDe MNs (arrows) from an animal ligated on day W0 a.m.. (B) TM-MFas II staining from the same animal shown in (A) labels the FeDe MNs (arrows). (C) Composite of A & B indicating that ligation on W0 a.m. does not prevent TM-MFas II expression by the FeDe MNs. (D) RDL backfill of the FeDe MNs (arrows) from an animal ligated on day L1. (E) TM-MFas II staining from the animal shown in (D). (F) Composite of A & B indicating that the FeDe MNs do not express TM-MFas II after ligation on day L1. (G) RDL backfill of the FeDe MNs (arrows) from an animal ligated on day L4 p.m. (H) TM-MFas II staining from the animal shown in (G). (I) Composite of G & H indicating that the FeDe MNs do not express TM-MFas II after ligation on L4 p.m. Scale bar = 100 μm in C (same magnification as in A & B), 100 μm in F (same magnification as in D & E), and 100 μm in I (same magnification as in G & H).

with the target muscle could lead to an upregulation of TM-MFas II-IR in the FeDe MNs, I severed the ventral nerve on the right side of metathoracic ganglia. The axon of the FeDe MN runs in the ventral nerve, along with the axons of the other leg MNs (Kent and Levine, 1988b). When I examined TM-MFas II-IR in these ganglia 4 days later, I found increased labeling in many cells ipsilateral to the nerve transection (Fig. 3B). In addition, only the FeDe MN ipsilateral to the nerve transection labeled with the TM-MFas II antibody (arrow, Fig. 3C). FeDe MNs from control preparations did not label with the TM-MFas II antibody (Fig. 3D-F). To test the specificity of this response, I severed the anterior connectives of metathoracic ganglia and examined the FeDe MNs 4 days later and found that this did not lead to increased TM-MFas II labeling in the FeDe MN (Fig. 3G-I). These results suggest that either damage to the FeDe MN's axon or a change in interactions with the larval FeDe muscle can upregulate TM-MFas II expression in the FeDe MN soma.

Intraganglionic JHM injection prevents upregulation of TM-MFas II expression by the FeDe MN

To determine if ecdysteroid-induced changes in the target muscle were sufficient to trigger increased TM-MFas II-IR in the absence of central ecdysteroid action, I injected JHM into larval metathoracic ganglia prior to the commitment pulse of ecdysteroids. This experimental manipulation prevents pupal commitment of the FeDe MN without affecting degeneration of the larval FeDe muscle (Knittel and Kent, 2002). Intraganglionic injections of oil did not prevent the normal increases in TM-MFas II-IR in the FeDe MN soma that is usually seen on day P1 (Fig. 4A-C). In contrast, injection of JHM into ganglia prevented the appearance of TM-MFas II-IR in the FeDe MNs (Fig.

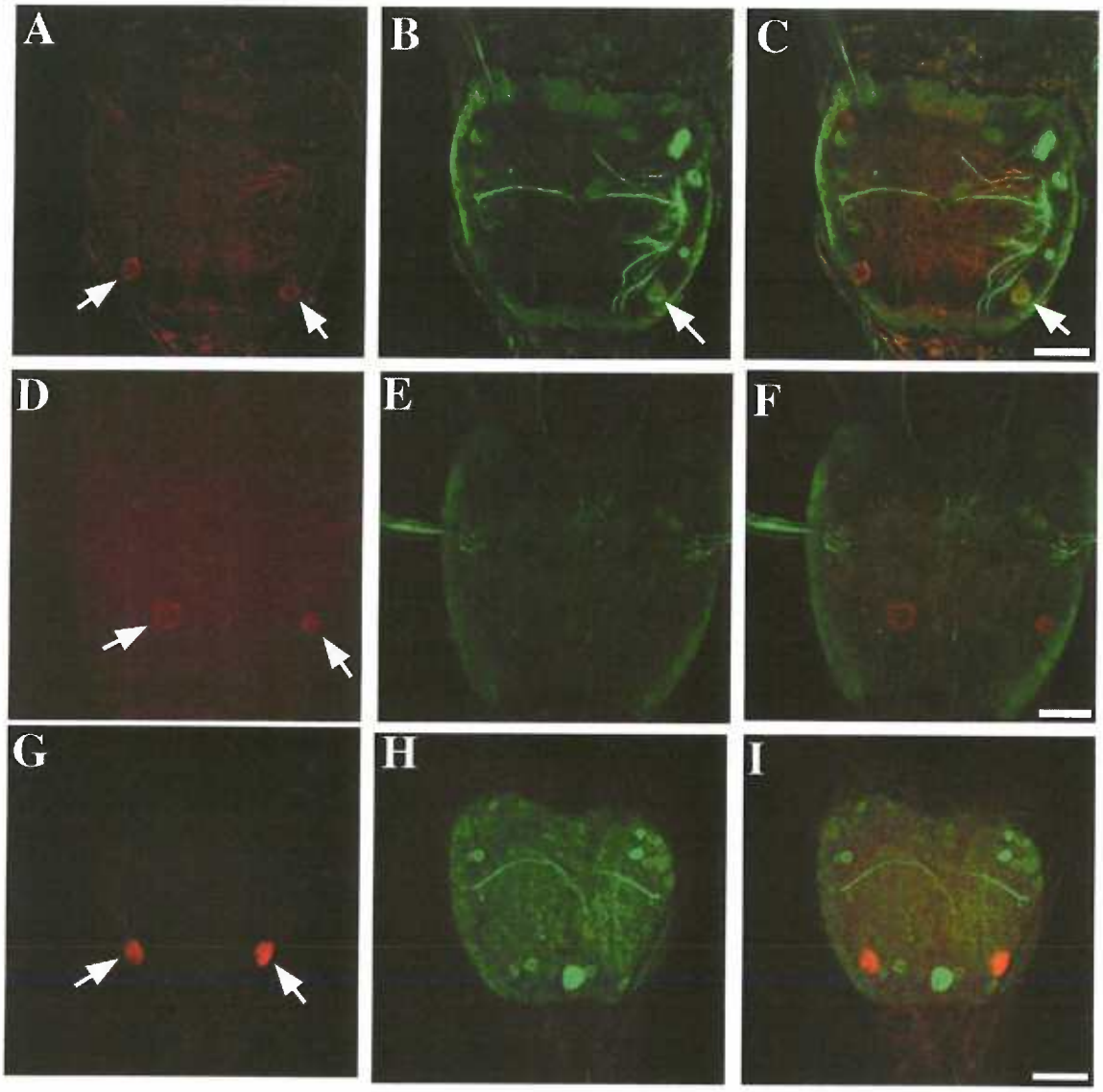

Figure 3. Expression of TM-MFas II by the FeDe MN in axotomized and control animals. (A-C) Right leg nerve axotomy, (D-F) unmanipulated control, and (G-I) axotomy of both anterior connectives. (A) RDL backfill of the FeDe MNs (arrows) from an animal that had the right leg nerve severed. (B) TM-MFas II staining from the same animal in (A) indicating that TM-MFas II-IR has increased on the right side of the ganglion. (C) Composite of A & B. (D) RDL backfill of the FeDe MNs (arrows) from an unmanipulated-control animal. (E) TM-MFas II staining from the animal in (D). (F) Composite of D & E showing that the FeDe MNs do not express TM-MFas II in unmanipulated animals of the equivalent stage. (G) RDL backfill of the FeDe MNs (arrows) from an animal that had both anterior connectives severed. (H) TM-MFas II staining of the animal in (G). (I) Composite of G & H indicating that severing the anterior connectives does not increase TM-MFas II-IR in the FeDe MN somata. Scale bar = 100 μm in C (same magnification as in A & B), 100 μm in F (same magnification as in D & E), and 100 μm in I (same magnification as in G & H).

4D-F). Therefore, in the absence of central ecdysteroid action during the commitment pulse, ecdysteroid-induced degeneration of target muscle or changes in nerve-muscle interactions are not sufficient to trigger the increase in TM-MFas II labeling seen in the FeDe MN.

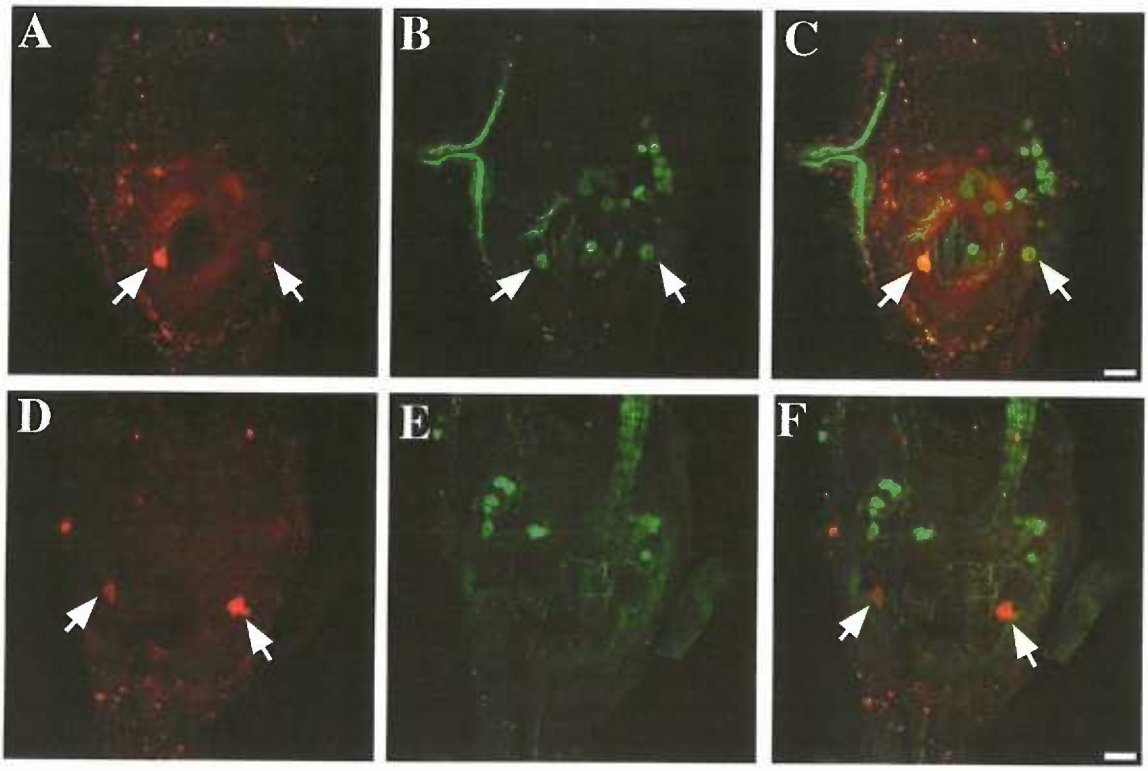

Figure 4. Expression of TM-MFas II by the FeDe MN at stage P1 in oil (A-C) and JHM-injected (D-F) ganglia. (A) RDL backfill of the FeDe MNs (arrows) from an animal that had oil injected into the metathoracic ganglion prior to the commitment pulse of ecdysteroids. (B) TM-MFas II staining from the same animal in (A) indicating that TM-MFas II-IR has increased throughout the entire ganglion. (C) Composite of (A & B) showing that the FeDe MNs have increased TM-MFas II-IR (arrows). (D) RDL backfill of the FeDe MNs (arrows) from an animal that had JHM injected into the metathoracic ganglion prior to the commitment pulse. (E) TM-MFas II staining from the animal in (D). (F) Composite of (D & E) showing that the FeDe MNs do no increase TM-MFas II-IR in JHM-injected animals (arrows). Scale bar = 100 μm in (C) [same magnification as in (A & B)], 100 μm in (F) [same magnification as in (D & E)].

DISCUSSION

In this study, I have confirmed that TM-MFas II-IR is not detectable in the FeDe MN soma prior to the rise in the prepupal stage. Preventing the commitment pulse of ecdysteroids through ligation on day L1 prevented the increase in detectable TM-MFas II-IR in the FeDe MN, although preventing the prepupal peak of ecdysteroids through ligation on the morning of day W0 did not. Ligation on the evening of day L4 was able to prevent the increase in detectable TM-MFas II-IR in the FeDe MN soma, possibly because ligation at this time cut the commitment pulse short. My results suggest that full exposure to the commitment pulse of ecdysteroids is necessary in order for the increase in TM-MFas II-IR to occur. My results also suggest that the ecdysteroids regulate the increase in TM-MFas II-IR in the FeDe MN through a direct action on the soma rather than indirectly through actions on the target muscle, at least if the MN is not yet pupally committed. Axotomy of the FeDe MN is an additional mechanism by which TM-MFas II-IR can be increased regardless of the commitment state of the MN. The fact that TM-MFas II-IR increases following nerve damage and during ecdysteroid-mediated remodeling suggest that TM-MFas II may play a role in mediating the FeDe MN's synaptic plasticity.

Regulation of TM-MFas II expression: role of the ecdysteroids

We have previously reported that TM-MFas II-IR is detectable in the FeDe MN soma and peripheral processes by stage W2 (Knittel et al., 2001), and this appearance of immunoreactivity coincides with the prepupal peak of ecdysteroids. In the current study, I have confirmed that the FeDe MN soma does not display TM-MFas II-IR prior to the rise in the prepupal peak of ecdysteroids by using double-label confocal microscopy. The

FeDe MNs from 5th instar or day W0 larvae do not display TM-MFas II-IR (Fig. 1C & 1F).

To investigate a possible role of the prepupal peak of ecdysteroids in regulating the appearance of TM-MFas II-IR in the FeDe MN soma, I ligated animals at 3 time points. I found that ligation on day L1 and L4 p.m. could both prevent the appearance of TM-MFas II-IR in the FeDe MN soma while ligation on W0 a.m. could not. On day W0, animals have experienced the commitment pulse of ecdysteroids but not the prepupal peak of ecdysteroids (Bollenbacher et al., 1981). The fact that ligations on day L4 p.m. could prevent the appearance of TM-MFas II-IR in the FeDe MN soma but that ligations on day W0 a.m. could not was surprising. Ligation on L4 p.m. did not prevent exposure of the dorsal vessel the next morning, which indicates that the animals had experienced the commitment pulse (Truman and Riddiford, 1974). This result suggests that different tissues became committed at different times during the commitment pulse such that exposure of the dorsal vessel requires a shorter exposure to the commitment pulse than the nervous system. Presumably, ligations on L4 shortened the commitment pulse such that the FeDe MN was not fully committed. By W0 a.m. the FeDe MN was fully committed, and a 4 day period was sufficient for expression of TM-MFas II to become detectable. Whether basal levels of ecdysteroids present at W0 played a role in the subsequent upregulation could not be determined from my experiments. During normal development, the FeDe MN in a stage W0 larva does not have detectable TM-MFas II-IR. Thus, the commitment pulse normally does not lead to TM-MFas II expression prior to the initial rising phase of prepupal ecdysteroids.

Very few studies have investigated the steroid regulation of cell adhesion molecule expression by MNs. Studies on the sexually dimorphic spinal nucleus of the bulbocavernosus in rats have indicated that testosterone upregulates expression of the calcium-dependent cell adhesion molecule (N-cadherin) in the MNs (Monks et al., 2001; Monks and Watson, 2001). Cell adhesion molecules can link the extracellular environment to the cytoskeleton (reviewed by Crossin and Krushel, 2000) and therefore may be involved in steroid-induced neuronal plasticity. The ecdysteroids regulate the dramatic restructuring of the nervous system that occurs during insect metamorphosis (Truman, 1996), and ecdysteroid regulation of TM-MFas II suggests a mechanism by which changes in the ecdysteroid titer could be translated into changes in neuronal morphology.

Regulation of TM-MFas II expression: role of axotomy

Transecting the leg nerve increased TM-MFas II-IR in the FeDe MN soma (Fig. 3A-C). This was a specific effect on the leg nerve and not due to damage to the ganglion in general, since severing the anterior connectives of the metathoracic ganglion did not increase TM-MFas II-IR (Fig. 3G-I). The increased TM-MFas II-IR in the FeDe MN soma was most likely a response to cellular damage (axotomy) rather than from loss of contact with the larval FeDe muscle. The finding that intraganglionic injections of JHM prevented the increase in TM-MFas II-IR suggests that degeneration of the target muscle is not sufficient, at least when the MN is not committed (Fig. 4D-F).

The role of axotomy in upregulating expression of Fas II has not been previously investigated. Expression of NCAM, the vertebrate homologue of Fas II, increases at the NMJ following nerve transection (Covault and Sanes, 1985). In addition, after a

complete transection of the spinal cord, NCAM is first expressed in MN somata and only later appears in axons (Tzeng et al., 2001). In my experiments, axotomy did not increase TM-MFas II-IR at the FeDe NMJ (data not shown), perhaps because preparations were not left long enough for reinnervation to occur.

Role of Fasciclin II in motor-terminal remodeling

Fas II has been shown to play a role in synaptic stabilization and growth at the *Drosophila* larval NMJ. However, a role for Fas II during the postembryonic remodeling of motor terminals has not been investigated. We have shown previously that intraganglionic injections of JHM prior to the commitment pulse of ecdysteroids prevents the final elimination of varicosities of retracted FeDe motor terminals (Knittel and Kent, 2002). Here, I show that the same intraganglionic injections of JHM prevent the increased TM-MFas II-IR normally seen during the prepupal stage. Possibly, increased TM-MFas II levels are needed for the final elimination of varicoscities during motor-terminal retraction, which is not the usual role of adhesion molecules during periods of synaptic plasticity. In *Drosophila*, a threshold amount of Fas II at the NMJ is required for synaptic stabilization, while a downregulation of Fas II is required for synaptic growth.

We have previously shown that during FeDe motor-terminal remodeling, the motor terminals express TM-MFas II while the peripheral glia that ensheath the FeDe motor terminals express GPI-MFas II (Knittel et al., 2001). The structure of *Manduca's* NMJ more closely resembles vertebrate NMJs where the nerve terminal is capped by an endplate glial cell. This is different from the structure of *Drosophila's* NMJ where the endplates are not capped by glial cells but rather are ensheathed by involutions of the

muscle membrane called the subsynaptic reticulum (Atwood et al., 1993). The intimate association of the FeDe MN's motor terminals with endplate glia could allow glia to play an active role in motor-terminal remodeling. Retraction of glial cell processes actually precedes withdrawal of the nerve during the metamorphic dismantling of the NMJs of flight muscle (Rheuben, 1992). Perhaps interactions with glial cells are required for motor-terminal retraction, and TM-MFas II expression by the FeDe MN is required for binding to GPI-MFas II on the glial cells. Peripheral ecdysteroid action may be necessary to initiate these glial changes.

In my experiments, ligation on day W0 a.m. did not prevent the upregulation of TM-MFas II in the FeDe MN soma but it did prevent expression in the motor-terminals (data not shown). However, increased TM-MFas II expression by the MN soma was not sufficient for motor-terminal retraction to occur since animals ligated on day W0 did not undergo motor-terminal retraction. How the peripheral distribution of TM-MFas II is regulated in *Manduca* is not known. Perhaps newly produced TM-MFas II had not yet been transported to the motor terminals at the time I dissected the ligated preparations. Peripheral interactions, either with glia or the muscle, could potentially regulate the distribution of peripheral TM-MFas II, and peripheral ecdysteroid action may be necessary to initiate these interactions.

Acknowledgements

I would like to thank Dr. Michael Danilchik for his assistance with the confocal microscopy and Dr. Philip Copenhaver for giving me the anti-TM-MFasclicin II antibody.

DISCUSSION

Summary and Conclusions

Through the experiments presented in this dissertation, I have examined the hormonal control of motor-terminal remodeling of an identified MN during metamorphosis of the moth, *Manduca sexta*. Using antisera against the synaptic vesicle protein synaptotagmin and against two isoforms of *Manduca* Fas II, I described the time course of the retraction of larval motor terminals and the growth of adult motor terminals. Next, I manipulated *Manduca's* hormonal environment to create heterochronic mosaic insects to investigate the respective roles played by ecdysteroid action on the CNS and the periphery in directing both motor-terminal retraction and motor-terminal growth. Finally, as a first step towards identifying local cues that may mediate motor-terminal remodeling locally, I investigated whether expression of the TM isoform of MFas II was regulated by the ecdysteroids acting either centrally or peripherally. My results were the following:

1. Retraction of FeDe motor terminals begins during the prepupal stage coincident with the rise in the prepupal peak of ecdysteroids.
2. Growth of FeDe motor terminals occurs during the pupal stage coincident with the pupal peak of ecdysteroids.
3. The GPI-linked isoform of MFas II is expressed by the glial cells that ensheath the peripheral nerves and endplate glia at the FeDe NMJ throughout the larval, pupal, and adult stage.
4. The TM isoform of MFas II is expressed by the FeDe MN only during the active period of motor-terminal remodeling.

5. Retraction of larval motor-terminals is controlled by interactions with peripheral cues, although the FeDe MN soma must experience the commitment pulse of ecdysteroids in order to respond fully to these cues.
6. Growth of adult motor terminals is controlled by interactions with the developing target muscle.
7. TM-MFas II expression by the FeDe MN soma during the prepupal stage is regulated by both central actions of the ecdysteroids and by nerve transection.

The results from my thesis support a model in which peripheral tissues, such as the FeDe muscle or the endplate glia, are the direct targets of the ecdysteroids, and motor-terminal remodeling is controlled by cell-cell interactions between the FeDe motor terminals and peripheral tissues. Commitment of the neuron by central ecdysteroid action, however, is necessary for specific aspects of remodeling. TM-MFas II is a molecule that is regulated by the ecdysteroids and therefore is a candidate likely to be involved in some aspects of motor-terminal remodeling. While commitment of the FeDe MN appears to be required for the upregulation of TM-MFas II in the MN soma, peripheral actions of ecdysteroids may regulate its subsequent peripheral distribution. My results suggest a mechanism by which peripheral actions of ecdysteroids could control motor-terminal remodeling, in part by regulating the peripheral expression or distribution of TM-MFas II once the MN has been committed by central actions of the ecdysteroids.

Role of cell-cell interactions in FeDe NMJ remodeling

My data suggests that remodeling of the FeDe MN's motor terminals is controlled locally via peripheral cell-cell interactions. Additionally, two isoforms of the cell adhesion molecule MFas II are expressed at the FeDe NMJ during motor-terminal remodeling, and the expression of TM-MFas II by the FeDe MN is regulated by the ecdysteroids. Although Fas II has been shown to regulate synaptic growth in *Drosophila* (Schuster, 1996a), a role for Fas II during postembryonic remodeling of motor terminals has not been described. My results indicate that TM-MFas II could play a role in remodeling of the FeDe NMJ. Although motor-terminal retraction and muscle death occurred when the upregulation of TM-MFas II was prevented, I found that lack of expression of TM-MFas II was associated with the incomplete loss of synaptic varicosities during motor-terminal retraction. Although I did not investigate the role of TM-MFas II during motor-terminal growth, my results combined with studies by other investigators suggest that Fas II is a potential candidate for mediating postembryonic motor-terminal growth (discussed below).

Besides Fas II, many other potential candidates exist for mediating cell-cell interactions between the FeDe MN's motor terminals and the target muscle or endplate glia. In *Drosophila*, the cell adhesion molecule Fas I has been shown to be involved in the fine tuning of motor-terminal arborizations (Zhong and Shanley, 1995). Another cell adhesion molecule, Fas III, seems to be involved in MN target recognition, since MNs can be forced to innervate muscles that misexpress Fas III (Kose et al., 1997). PS integrin expression at the *Drosophila* larval NMJ can regulate growth and morphology of motor terminals, and this effect could be due to a disruption in the normal cis interactions

of integrins with other cell adhesion molecules, such as the fasciclins, or due to disruption of integrin-mediated second messenger pathways, such as the activation of the CaM kinase II cascade (Beumer et al., 1999). The secreted glycoprotein semaphorin II is a negative regulator of MN synapse formation following target recognition in *Drosophila* (Matthes et al., 1995). Finally, mutations in neuroglian, which is the invertebrate homologue of the vertebrate cell adhesion molecule L1, affect the specificity of NMJ formation during embryogenesis in *Drosophila*, although neuroglian was not required during late larval and pupal stages (Hall and Bieber, 1997). Thus, many molecules control the specificity of synapse formation and the morphology of motor terminals at the NMJ, and therefore it is likely that combinations of molecules rather than just one molecule are involved in remodeling of the FeDe NMJ.

Role of ecdysteroids in FeDe NMJ remodeling

My results indicate that the ecdysteroids control the retraction of the FeDe MN's motor terminals indirectly through an action on the target muscle, and the FeDe MN soma must experience the commitment pulse of ecdysteroids in order to respond fully to peripheral cues (chapter 2). Although I did not demonstrate that the FeDe muscle is a direct target of the ecdysteroids, previous investigators have shown that other remodeled insect muscles are direct targets (Hegstrom et al., 1998; Zachary and Hoffmann, 1980). Previous studies have also found that motor-terminal retraction is a local response to muscle degeneration, although those studies did not investigate the respective roles of the commitment pulse and the prepupal peak of ecdysteroids, nor did they examine the motor-terminals and dendrites from the same MN in order to evaluate the MN soma's ecdysteroid exposure (Hegstrom, 1996b). Finally, I was able to determine that dendritic

regression and motor-terminal retraction are separable events that must be regulated by different mechanisms.

I found that during growth of the FeDe MN's adult motor terminals, interactions with the developing target muscle are sufficient for the elongation of axonal arbors and the differentiation of adult endplates (chapter 3). Previous studies had suggested that the elongation of axonal arbors was initiated by exposure of the MN soma to ecdysteroids while interactions with the target muscle controlled the formation of transverse sprouts and the differentiation of synaptic boutons (Truman and Reiss, 1995). Those studies utilized either systemic or local peripheral applications of JHM, which produced regrown larval target muscles, and therefore the effects observed could have been due to motor-terminal interactions with an abnormal target muscle (Truman and Reiss, 1995). Moreover, their experiments did not address the possibility that interactions with a normal target muscle might also be sufficient for motor-terminal elongation. The heterochronic mosaic insect I created that utilized intraganglionic injections of JHM to block the central effect of the ecdysteroids allowed me to test the effects of central and peripheral ecdysteroids in a different way. I was able to block the central effect of the ecdysteroids while allowing a normal target muscle to form. In this way, I was able to ask if interactions with the developing adult FeDe muscle were sufficient for both motor-terminal growth and endplate formation. I also examined the motor-terminals and the dendrites from the same MNs to evaluate the MN soma's ecdysteroid exposure. As I discovered for the regressive aspect of FeDe MN remodeling, dendritic growth and motor-terminal growth are separable events and therefore are regulated by different mechanisms.

Mechanisms of locally-mediated neurite outgrowth

The evidence to date suggests that the ecdysteroids induce metamorphic restructuring of MN processes by initiating gene transcription in the MN soma. *In vitro* studies on cultured leg MNs revealed that in the presence of 20E, neurons grew more higher-order branches (Prugh, 1992) and had more complex growth cone structure (Matheson and Levine, 1999), suggesting that 20E may induce transcription of genes involved in process outgrowth. In the rat SNB, expression of mRNAs for β-actin and β-tubulin decreases in castrated rats, and this decrease is prevented by testosterone treatment (Matsumoto et al., 1994). Therefore, transcription of genes for cytoskeletal proteins might underlie testosterone-induced plasticity of MN morphology. Similarly, in *Manduca*, a genomic action of 20E on remodeled MNs has been suggested to underlie the rapid longitudinal growth of axons across developing adult muscle (Truman and Reiss, 1995). The results of my thesis indicate that local interactions with the target muscle are also sufficient for inducing the longitudinal growth of the FeDe MN motor terminals (chapter 3).

Locally-stimulated neurite outgrowth could occur via a number of mechanisms. NCAM stimulates neurite outgrowth *in vitro* through second messenger cascades involving activation of the FGF receptor, calcium influx, and CaM kinase activation (reviewed by Walsh and Doherty, 1997). This NCAM/FGF-stimulated neurite outgrowth causes phosphorylation of GAP-43 and does not occur in mutants lacking GAP-43 (Meiri et al., 1998). GAP-43 expression has also been shown to induce synaptic growth *in vivo* at the vertebrate NMJ (Caroni et al., 1997). The Rac subfamily of GTPases seem to be involved in axon outgrowth in *Drosophila*, and mutations in Drac1 cause defects in axon

outgrowth and are associated with defects in F-actin distribution in growth cones (Luo et al., 1994). Finally, the diffusible molecule NO has been implicated as a candidate for shaping the morphology of axonal arbors in vertebrates (Gally et al., 1990) and invertebrates (Seidel and Bicker, 2000). Clearly, many mechanisms exist for eliciting neurite outgrowth besides steroid-induced transcription of genes for cytoskeletal proteins. Growth of the FeDe MN's adult motor-terminals may utilize one such mechanism.

Fas II is a molecule with the potential to mediate locally-induced neurite outgrowth of the FeDe MN. Although signal transduction cascades initiated by Fas II homophilic binding have not been identified, Fas II is the invertebrate homologue of NCAM and regulates synaptic growth at the *Drosophila* larval NMJ (Schuster, 1996a). Differential expression of Fas II is the main determinant of synaptic growth discovered so far (Schuster, 1996a). A downregulation of Fas II is sufficient for the structural aspects of synaptic growth to occur (Schuster et al., 1996b) while a concurrent expression of CREB (the cAMP response element binding protein) is responsible for the transcription of genes required for increasing synaptic strength (Davis et al., 1996). In *Aplysia*, a homologue of NCAM called apCAM regulates synaptic growth through an endocytosis-mediated internalization of the TM isoform of apCAM (Bailey et al., 1997). Because the TM isoform of MFas II is expressed at the FeDe NMJ during the time that motor-terminal growth is occurring (chapter 1) and has been shown to mediate motor-terminal growth in other systems, TM-MFas II may mediate motor-terminal growth at the FeDe NMJ. Expression of TM-MFas II is upregulated, rather than downregulated, during motor-terminal remodeling in *Manduca*, suggesting potential novel mechanisms and roles during metamorphosis. Perhaps an initial upregulation of TM-MFas II is required for its

distribution to the motor terminals and then subsequent more subtle fluctuations in Fas II levels at the growing tips locally regulate neurite outgrowth.

FUTURE DIRECTIONS

The results of my thesis study suggest that remodeling of the FeDe MN's motor terminals during metamorphosis is controlled by local cell-cell interactions between the motor terminals and peripheral tissues. In addition, I identified the cell adhesion molecule MFas II as being present at the FeDe NMJ during the time that motor-terminal remodeling occurs. Many new questions have been revealed by this study. First of all, the relationship between the three cellular components of the NMJ during metamorphic remodeling needs to be investigated. Second, they hypothesis that peripheral ecdysteroid actions control peripheral expression of TM-MFas II needs to be investigated further. Third, the hypothesis that MFas II is a local cue that plays a role in motor-terminal remodeling needs to be tested. Finally, additional molecules that could mediate local cell-cell interactions at the FeDe NMJ need to be identified.

A closer examination of the FeDe NMJ during metamorphosis to determine the precise role of glia may provide insight into the mechanisms underlying motor-terminal remodeling. *Manduca's* neuromuscular junction is similar to vertebrate neuromuscular junctions in that the nerve terminal is capped by an endplate glial cell. This is in contrast to the *Drosophila* NMJ where the nerve terminals are not capped by glial cells but rather are ensheathed by involutions of the muscle membrane (Atwood et al., 1993). Previous studies on flight muscle have indicated that withdrawal of the endplate glia from the NMJ actually precedes nerve retraction (Rheuben, 1992). My results indicated that the glial cells that ensheath the FeDe MN axon and endplates express GPI-MFas II throughout *Manduca's* metamorphic transformation (chapter 1). Glial expression of GPI-MFas II at the FeDe NMJ could be merely involved in glial-glial adhesion, or perhaps GPI-MFas II-

expressing glial cells could adhere to the TM-MFas II-expressing FeDe motor nerve during retraction and growth. Ultrastructural studies on the FeDe NMJ could reveal if the glial cells are the first cellular component to undergo changes in response to ecdysteroid exposure as had been found for flight muscle (Rheuben, 1992). In addition, ultrastructural studies could reveal if during motor-terminal growth the glial cells follow the FeDe MN growth cone or if the glial cells pattern the pathway for the MN. Perhaps the endplate glia play an active role in remodeling of FeDe motor terminals through GPI-MFas II to TM-MFas II adhesive interactions. One test of this hypothesis would involve injecting phospholipase C into larval and pupal legs to cleave the GPI-MFas II from the membrane to determine if motor-terminal remodeling would occur in the absence of this adhesive interaction.

My results indicated that peripheral ecdysteroid actions may control peripheral expression or localization of TM-MFas II. Animals ligated on W0 a.m. increased TM-MFas II expression in FeDe MN somata, but not in FeDe motor terminals (chapter 4). In these animals, ligation on W0 a.m. prevented degeneration of the FeDe muscle, leaving open the possibility that peripheral ecdysteroid actions may be involved in peripheral expression or localization of TM-MFas II. To test the hypothesis that peripheral ecdysteroid actions lead to peripheral expression or localization of TM-MFas II, RH5992 could be injected into the legs of animals ligated on day W0 a.m. The RH5992 injection would trigger degeneration of the FeDe muscle (chapter 2), and these muscles could be examined for TM-MFas II-IR in FeDe motor terminals. If TM-MFas II-IR was detected in FeDe motor terminals, then this result would suggest that once the MN is pupally committed, peripheral actions of ecdysteroids are sufficient for expression of TM-MFas

II in the periphery.

To test the hypothesis that TM-MFas II is a local cue that plays a role in motor-terminal remodeling, it would be necessary to manipulate TM-MFas II levels *in vitro* or *in vivo* to reveal if any aspect of motor-terminal remodeling could occur in its absence. Function-blocking antibodies to TM-MFas II could be injected into larval or pupal legs, and the FeDe muscle could be evaluated later in development to determine if any aspect of motor-terminal remodeling had been affected. The role of TM-MFas II during motor-terminal growth could be tested using an *in vitro* model through coculture of myocytes with MNs. Previous studies have shown that in the presence of MNs, isolated myocytes will fuse to form myotubes, and the MNs will make synaptic contact with the myotubes (Luedeman, 1996). Using this paradigm with the FeDe MN, the MNs and myocytes could be incubated in the presence of function-blocking antibodies to determine if interruption of MFas II adhesion affects nerve-muscle interactions.

Although my results have indicated that MFas II is expressed at the FeDe NMJ during the correct time to play a role in FeDe motor-terminal remodeling, it is most likely not the only local cue involved. Indeed, when the upregulation of TM-MFas II by the FeDe MN soma is prevented, most aspects of motor-terminal retraction occur normally (chapters 2 & 4). During motor-terminal retraction, however, the FeDe MN must experience the commitment pulse of ecdysteroids in order for the final elimination of synaptic varicosities to occur. Thus, the upregulation of TM-MFas II may be required. Alternatively, many molecules with the potential to influence synaptic morphology have been identified. Therefore, antibodies against those proteins could be used to determine if any of them is expressed at the FeDe NMJ. If any of those molecules are found to be

expressed at the FeDe NMJ, then function-blocking antibodies could be injected into larval and pupal legs to determine if interruption of their function has an influence on motor-terminal remodeling.

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