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Hormone-Dependent Expression of Fasciclin II During Ganglionic Migration and Fusion in the Ventral Nerve Cord of the Moth *Manduca sexta*

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Abstract

The ventral nerve cord of holometabolous insects is reorganized during metamorphosis. A prominent feature of this reorganization is the migration of subsets of thoracic and abdominal larval ganglia to form fused compound ganglia. Studies in the hawkmoth *Manduca sexta* revealed that pulses of the steroid hormone 20-hydroxyecdysone (20E) regulate ganglionic fusion, but little is known about the cellular mechanisms that make migration and fusion possible. To test the hypothesis that modulation of cell adhesion molecules is an essential component of ventral nerve cord reorganization, we used antibodies selective for either the transmembrane isoform of the cell adhesion receptor fasciclin II (TM-MFas II) or the glycosyl phosphatidylinositol-linked isoform (GPI-MFas II) to study cell adhesion during ganglionic migration and fusion. Our observations show that expression of TM-MFas II is regulated temporally and spatially. GPI-MFas II was expressed on the surface of the segmental ganglia and the transverse nerve, but no evidence was obtained for regulation of GPI-MFas II expression during metamorphosis of the ventral nerve cord. Manipulation of 20E titers revealed that TM-MFas II expression on neurons in migrating ganglia is regulated by hormonal events previously shown to choreograph ganglionic migration and fusion. Injections of actinomycin D (an RNA synthesis inhibitor) or cycloheximide (a protein synthesis inhibitor) blocked ganglionic movement and the concomitant increase in TM-MFas II, suggesting that 20E regulates transcription of TM-MFas II. The few neurons that showed TM-MFas II immunoreactivity independent of endocrine milieu were immunoreactive to an antiserum specific for eclosion hormone (EH), a neuropeptide regulator of molting.

Keywords

20-hydroxyecdysone; cell adhesion molecule; eclosion hormone; interstitial axonal growth; neurometamorphosis; pterothoracic ganglion

During metamorphosis, the ventral nerve cord of holometabolous insects undergoes a dramatic reorganization: individual segmental ganglia fuse to form larger compound ganglia (Pipa, 1969; Amos and Mesce, 1994). This phenomenon has been studied primarily in

lepidopterans (Pipa and Woolever, 1964, 1965; Pipa, 1967, 1969; Coulon, 1979; Singh and Singh, 1980; Eaton, 1988; Amos and Mesce, 1994; Cantera et al., 1995; Amos et al., 1996) but also in the fruit fly *Drosophila melanogaster* (Takaki and Sakuri, 2003; Olofsson and Page, 2004) and a stingless bee, *Melipona quadrifasciata* (Pinto et al., 2003).

In the hawkmoth *Manduca sexta*, the most prominent event of nerve cord reorganization occurs early in the transition between the larval and the pupal stages. At this time, the second and third thoracic ganglia fuse with the first and second abdominal ganglia. Each of these ganglia travels a significant distance from its original location to form the pterothoracic ganglion (Amos and Mesce, 1994). Because the pterothoracic ganglion contains the flight pattern generator and integrates flight-related sensory inputs, it has been argued that ganglionic consolidation facilitates flight by permitting the formation of novel synaptic contacts in the central neuropil of the fused ganglion and by reducing interganglionic conduction times (Robertson and Pearson, 1985; Altman and Kien, 1987; Duch and Pflüger, 1999). The terminal abdominal ganglion of the adult central nervous system (CNS) is formed by a similar process of migration and fusion (A6 fuses with the larval terminal ganglion) slightly later in the process of metamorphosis.

At the cellular level, ganglionic migration and fusion are striking phenomena. Axons coil within shortened interganglionic connectives and elongate in nerve roots, whereas packets of neuronal somata shed their ganglionic sheaths to migrate toward adjacent ganglia. These processes culminate in the fusion of adjacent ganglia (Amos and Mesce, 1994; Amos et al., 1996). Only subsets of thoracic and abdominal ganglia migrate and fuse; others remain relatively stationary, indicating that some aspect of segmental identity regulates ganglionic movement (Amos and Mesce, 1994; Fahrbach et al., 2001). Ganglionic migration and fusion are controlled by the sequential pulses of the steroid hormone 20-hydroxyecdysone (20E) that signal the beginning of metamorphosis (Amos et al., 1996). Two pulses are critical: the prepupal peak at the end of larval life and the subsequent preadult rise that begins shortly before the day of pupal ecdysis. Both the decline of the prepupal peak and the onset of the preadult rise are necessary and sufficient for ganglionic migration to initiate and proceed normally (Amos et al., 1996).

How neurons within fusing ganglia move to their new locations is unknown. The somata travel in tight clusters in the appropriate direction. It has been suggested that neurons per se are not responsible for their own motility but rather that glial cells tow and/or push the neurons to their new locations (Pipa and Woolever, 1964, 1965; Pipa, 1967; Amos and Mesce, 1994; Fahrbach et al., 2001). An understanding of neuronal–glial interactions during nerve cord reorganization has been hampered by the relative paucity of glial-specific markers in insects (Carlson and Marie, 1990; Loesel et al., 2006). We therefore sought an alternative approach to study the process of nerve cord reorganization. Reasoning that neurons that migrate in clusters to new positions likely modulate their adhesion to neighboring neurons and glial cells, we initiated studies of insect cell adhesion molecules (CAMs) in the context of ventral nerve cord reorganization (Tessier-Lavigne and Goodman, 1996; Wright et al., 1999; Wright and Copenhaver, 2000). We examined the spatial and temporal profiles of fasciclin II, an insect-specific CAM, because it has been implicated in numerous events in the developing insect nervous system, including neuronal recognition, axon fasciculation, and growth cone guidance during embryogenesis (Grenningloh et al., 1991; Lin et al., 1994; Lin and Goodman, 1994). In both the larval and the mature nervous systems, fasciclin II has additional actions at the synapse related to activity-dependent synaptic plasticity (Schuster et al., 1996a,b; Davis et al., 1997; Goodman et al., 1997; Baines et al., 2002; Sigrist et al., 2003).

Fasciclin II was identified and cloned from the CNS of the grasshopper (Bastiani et al., 1987; Harrelson and Goodman, 1988; Snow et al., 1988). It is a homophilic cell adhesion molecule and a member of the immunoglobulin (Ig)-related superfamily of CAM receptors, characterized by an extracellular domain containing five Ig-like C2 domains and two fibronectin type III domains (Grenningloh et al., 1990, 1991; Brummendorf and Rathjen, 1993). Fasciclin II is structurally similar to the vertebrate receptor NCAM (Cunningham et al., 1987; Grenningloh et al., 1990) and *Aplysia* apCAM (Mayford et al., 1992). Two isoforms of fasciclin II have been cloned from *Manduca sexta* (Wright et al., 1999). One 95-kDa isoform spans the cell membrane and has an intracellular carboxy-terminus (TM-MFas II); the other (90 kDa) is attached to the extracellular surface of the cell membrane by a glycosyl phosphatidylinositol anchor (GPI-MFas II). These isoforms of fasciclin II have also been identified in other species of insects, including the grasshopper *Schistocerca americana* and the fruit fly *Drosophila melanogaster* (Snow et al., 1988; Grenningloh et al., 1991; Lin and Goodman, 1994).

Several previous studies in *Manduca sexta* revealed broad differences in the distribution of the two isoforms of fasciclin II within the CNS. TM-MFas II is expressed by migrating neurons and their processes during formation of the enteric nervous system (Wright et al., 1999; Wright and Copenhagen, 2000, 2001). Modulated expression of TM-MFas II was also observed on the axon terminals of identified *Manduca sexta* leg motoneurons that retract and regrow during the neuromuscular remodeling associated with metamorphosis as well as on a subset of ingrowing olfactory receptor neurons during their initial innervation of the antennal lobe glomeruli (Knittel et al., 2001; Higgins et al., 2002). In contrast, GPI-MFas II is expressed primarily by glial cells associated with the midgut and glia that ensheath peripheral neurons, including the transverse nerve of the segmental ganglia (Wright and Copenhagen, 2000).

Here we report the distribution of the TM and GPI isoforms of fasciclin II in the ventral nerve cord of *Manduca sexta* during the larval–pupal transition and the subsequent period of adult development. Because it is possible that one of the functions of TM-MFas II is to serve as a CAM mediating the adhesion needed to package neurons so that they can be moved as ensembles, we predicted (1) that neuronal somata and axons would express TM-MFas II during ventral nerve cord reorganization and not during periods of morphological stability, (2) that migrating ganglia would express TM-MFas II more abundantly than nonmigrating ganglia, and (3) that hormonal manipulations that block migration and fusion would reduce TM-MFas II expression. We anticipated that GPI-MFas II immunoreactivity might serve as a novel marker for tracking changes in glial cell populations during ventral nerve cord reorganization.

MATERIALS AND METHODS

Animals

Larvae of the tobacco hornworm *Manduca sexta* (Lepidoptera: Sphingidae) were reared individually on an artificial diet (Bell and Joachim, 1976). Larvae were maintained at 27°C and 50–60% relative humidity on a 17:7-hour light:dark cycle (LD17:7). At the beginning of the 18-day period of adult development, pupae were exposed to a temperature shift from 27°C during the day to 25°C at night to improve synchrony of adult ecdysis (Lockshin et al., 1975). Developing pupae and pharate adults were staged according to Tolbert et al. (1983) and Amos and Mesce (1994). Under our rearing conditions, each stage is roughly equivalent to 1 day. The age of larvae is given by the number of the larval instar, with W0 designating the first day of the nonfeeding, wandering phase of the fifth and final larval instar. The thinning of the cuticle over the dorsal vessel and stereotypical locomotory behavior were used as markers for wandering (Reinecke et al., 1980). The developmental stage of pupae is

described in relation to the shedding of the larval cuticle at the end of the larval–pupal molt, an event referred to as *pupal ecdysis*. The day of pupal ecdysis is designated P0. The age of adult animals is similarly described in relation to the emergence of the adult moth from the pupal cuticle (*adult ecdysis*). Both male and female animals were used in these studies.

Terminology

Thoracic and abdominal ganglia are numbered in sequence from anterior to posterior (T1, T2, T3, A1, A2, A3 etc.). The paired bundles of fibers that connect adjacent ganglia are composed of axons of intersegmental neurons and the axons of peripheral sensory neurons. They are referred to as *connectives* and are named by giving the more anterior of the two ganglia joined by a connective first: for example, the T3–A1 connective joins the third thoracic and the first abdominal ganglion. We use the term *migration* specifically to refer to the movement of neuronal somata and their trailing processes to form a compound ganglion. For convenience, we sometimes refer to the preparation that results when a larva or pupa is ligated immediately posterior to the prothoracic segment as an *isolated abdomen*, although such preparations also include the meso- and metathoracic body segments.

Immunocytochemistry

Insects were cooled on ice until unresponsive. The brain and ventral nerve cord were dissected in 4°C saline (150 mM NaCl, 5 mM KCl, 4 mM CaCl₂·2H₂O, 28 mM glucose, 5 mM HEPES). Dissected brains and ventral nerve cords were placed directly into 4% paraformaldehyde (Fisher Scientific, Pittsburgh, PA) prepared in phosphate-buffered saline (10 mM, pH 7.6, hereafter referred to as *PBS*). Fixation was performed at room temperature. Tissue from larvae was fixed for 1–4 hours; tissue from pupae and adults was fixed slightly longer (2.5–5 hours). After fixation, tissue was washed in PBS for 1 hour at room temperature or overnight at 4°C, then treated for 30 minutes with collagenase (type IV, 0.5 mg/ml) prepared in 10 mM PBS containing 1 mM CaCl₂. Tissue was then transferred to a blocking buffer containing 10% normal goat serum (NGS) and 1% Triton X-100 in PBS for 2–5 hours at room temperature. Unless otherwise specified, all reagents were obtained from Sigma Aldrich (St. Louis, MO). Because of the fragile nature of the ventral nerve cord during metamorphosis, all incubations and washes were performed without agitation.

For detection of fasciclin II, the brain and ventral nerve cord were incubated for 72–100 hours at 4°C in a 1:1,000 dilution (in PBS) of one of two guinea pig polyclonal antisera generated against specific segments of the two MFas II isoforms (gift of P.F. Copenhaver, Oregon Health Sciences University, Portland, OR). After incubation in a primary antiserum, tissue was washed in PBS for 1–5 hours and then incubated with a 1:200 dilution (in PBS) of donkey anti-guinea pig IgG conjugated to the fluorophore Cy5 (Jackson Immunoresearch, West Grove, PA) at 4°C for at least 50 hours.

For identification of eclosion hormone (EH) neurons, ganglia were incubated in a 1:100 or 1:250 dilution (in PBS) of a polyclonal antiserum generated in rabbit against bioactive material from the corpora cardiaca-corpora allata (CC-CA) complex of *Manduca sexta* (gift of J.W. Truman, University of Washington, Seattle, WA). Tissue was incubated in the primary antibody for 48–100 hours at 4°C, then washed in PBS for 1–5 hours, followed by incubation in a 1:200 dilution (in PBS) of goat anti-rabbit IgG conjugated to the fluorophore Cy3 (Jackson Immunoresearch) for at least 48 hours at 4°C. After incubation with the appropriate secondary antibody, all tissue was washed in PBS overnight at room temperature, dehydrated through an ethanol series, cleared in methyl salicylate, and mounted between two coverslips (No. 1, 22 × 40 mm) in DEPEX (EMS, Fort Washington, PA).

Characterization of antisera

The polyclonal guinea pig antisera used to detect individual isoforms of MFas II were generated against peptide conjugates corresponding to unique amino acid sequences found in the two MFas II isoforms. The TM-MFas II antiserum was generated against the sequence (C)TGE-DAIKRNSSVEFDGHRV. The GPI-MFas II antiserum was generated against the sequence (C)GEYNS-ESNEVPRQPGFYDV, which corresponds to the unique extracellular domain located adjacent to the GPI anchor site. The selective nature of each antiserum has been established by protein immunoblot analysis and by preadsorption controls using the peptides against which the antibodies were generated and was confirmed by comparison with the results of whole-mount in situ hybridization histochemistry using isoform-specific probes (Wright and Copenhaver, 2000). As expected, immunolabeling with these antibodies was restricted to the cell surface. The specificity of the antiserum used to identify EH neurosecretory neurons was confirmed by preadsorption of the working dilution of the antibody with a sample of EH prepared from CC-CA complexes of pharate adults as previously described (Terzi et al., 1988): each of these neurohemal organs contains about 3.5 ng of EH. The pattern of labeling obtained in the current experiment exactly matched that expected, based on published descriptions of the unique location and morphology of the ventromedial (VM) neurons, which have been independently verified by in situ hybridization histochemistry (Truman and Copenhaver, 1989; Riddiford et al., 1994). Therefore, although purified synthetic EH was not available for preadsorption, we are confident in our identification of the two pairs of VM neurons and their associated projection to the proctodeal nerve. The identity of several other neurosecretory neurons that were also EH immunoreactive (ir) in the present study requires further investigation, although EH immunoreactivity and bioactivity have been previously reported in these neurons (Copenhaver and Truman, 1986).

Imaging and fiber counting

Neurons within whole-mounted ganglia were initially examined with a Bio-Rad MRC-1024 laser scanning confocal microscope (Bio-Rad Laboratories, Hercules, CA) attached to a Nikon Diaphot inverted microscope equipped with a 15-mW krypton/argon laser. The Cy3 and Cy5 fluorophores were imaged at 568 nm and 647 nm excitation, respectively, paired with bandpass emission filters that transmitted light from 589–621 nm (Cy3) or 664–696 nm (Cy5). For high-magnification confocal images with maximal resolution, we used a Nikon C1si laser attached to a Nikon TE2000-E2 fully motorized DIC inverted microscope. The Cy5 fluorophores were imaged using 638 nm excitation. Confocal Assistant 4.02 or 5.2 software (T. C. Brelje, University of Minnesota; <http://www.bipl.umn.edu>) was used to produce a full projection of a stack of optical sections (taken every 1–5 μ m) as previously described (Mesce et al., 1993). In a given projection (z-series), the retention of very fine details that were shadowed by overlying optical sections was obtained by manually adjusting the contrast in these areas (Mesce et al., 1993). Projections and single images were then imported into Adobe Photoshop CS2 (version 9.0.2) for photographic assembly (Adobe Systems, Mountain View, CA); only changes to overall image brightness and contrast were subsequently made when needed.

The number of TM-MFas II-ir fiber tracts in the connectives between the third thoracic and the first abdominal ganglia (T3–A1) was determined as follows. First, a line was drawn orthogonal to the T3–A1 connectives in the area of the connective immediately adjacent to A1. Two observers independently counted the number of TM-MFas II-ir fiber tracts in the paired connectives under that line only; the two counts were then averaged. Because it was not always possible to distinguish between a single axon and a bundle of tightly fasciculated axons, the terms *fiber* (referring to a single axon) and *fiber tract* (referring to multiple, tightly fasciculated axons) are used interchangeably throughout this report. For fiber

counting, the laser iris was set at 10%, 30%, or 100%, depending on the intensity of label within the sample being examined. Varying the iris setting ensured that all TM-MFas II-ir fibers were counted regardless of the intensity of the label in a particular sample. It is important to note that the fiber counts reported here almost certainly underestimate the number of individual axons expressing TM-MFas II in the T3–A1 connective. For example, the two tightly fasciculated axons of the VM neurons known to be present in each hemiconnective have the appearance of a single fiber and were therefore counted as one fiber (Truman and Copenhaver, 1989).

Hormone manipulations

In initial studies designed to investigate the general relationship between titers of the steroid hormone 20E and TM-MFas II expression, the body posterior to the prothoracic segment was isolated from the head and anterior thorax at 1–2 hours after pupation on P0. This procedure, referred to as *ligation*, was performed on pupae with hemostatic forceps. In ligated animals, the body posterior to the clamped forceps was isolated from the prothoracic glands, which are the source of the 20E precursor, ecdysone (Warren et al., 1988). These preparations experienced the prepupal peak of 20E but not the later preadult rise. Animals ligated on P0 were reared at 27°C and 50–60% relative humidity under LD17:7.

In subsequent studies designed to prevent the ventral nerve cord from being exposed to both the prepupal peak of 20E and the subsequent preadult rise of 20E, larvae were ligated posterior to the prothoracic segment on W0 with cotton thread. Ligated larvae were maintained at room temperature (21–24°C; LD10:14), and the ventral surface of these preparations was routinely covered with petroleum jelly to minimize dehydration and increase survival. Cuticular markers were monitored to match the relative developmental and endocrine status of ligated larvae selected for study (Amos and Mesce, 1994).

In additional experiments designed to prevent the preadult rise of 20E but not the earlier prepupal peak, larvae within 24 hours of the larval–pupal transition were ligated (on W3) posterior to the prothoracic segment with cotton thread. Because such animals had already experienced the endogenous prepupal pulse of 20E, the isolated abdomens formed pupal cuticle on the next day.

Infusion and injection protocols used for ecdysteroid replacement were based on the methods of Amos et al. (1996). For all infusions, 20E (Sigma Aldrich) was dissolved in physiological saline (Ephrussi and Beadle, 1936) and infused with PE-10 polyethylene tubing (Curtin Matheson, Eden Prairie, MN). For infusion experiments, 20E was delivered by a microinfusion pump (model 352; Sage Instruments, Cambridge, MA) and a 100- μ l glass syringe (Hamilton, Reno, NV). The concentration of 20E was checked spectrophotometrically as described by Amos et al. (1996) and adjusted to a final concentration of 1.3 μ g/ μ l.

To mimic the prepupal peak, 20E was infused at a rate of 2.0 μ l per hour for 22 hours into larval abdomens ligated on W0. In these preparations, polyethylene tubing was inserted into the larval dorsal horn and held in place with cyanoacrylate. Four to five days later, 11 of 19 isolated larval abdomens exposed to this treatment formed pupal cuticle.

To mimic the preadult rise, the pupal cuticle of unmanipulated pupae was allowed to darken and harden (tan) for 2–10 hours following pupal ecdysis. Then, PE-10 tubing was inserted through the dorsal surface of the pupal cuticle at the level of A3, and 20E was infused at a concentration of 1.3 μ g/ μ l at a rate of 2.0 μ l per hour for 72 hours. Nerve cords were dissected immediately upon cessation of the infusion or with a delay of up to 12 hours, as

required by the protocol of the given experiment. Fifteen of twenty infused pupal abdomens survived the 72-hour infusion and were available for analysis.

We also performed sequential double infusions designed to mimic both the prepupal peak and the preadult rise. Thirty of sixty-one animals ligated on W0 that received a prepupal-peak infusion formed a pupal abdomen, but only 16 of these 30 pupal abdomens were judged healthy enough to receive the preadult rise infusion. Seven pupal abdomens that survived the sequential hormone treatments were available for analysis.

In some experiments, single injections of 20E were used to manipulate hormone titers, because the survival of injected insects was better than that of infused insects. To mimic the prepupal peak, ligated larvae were chilled on ice for 2 minutes and subsequently injected in the ventral thorax with a single, weight-dependent dose of 20E (15 $\mu\text{g/g}$ body weight, 1 $\mu\text{g}/\mu\text{l}$ 20E; as in Schactner et al., 2004). Fifteen of twenty-four abdomens that received this treatment formed pupal cuticle; these animals were available for analysis. To mimic the preadult rise, abdomens isolated on W3 that formed pupal cuticle were injected 2–3 hours postecdysis with a single, weight-dependent dose of 20E (15 $\mu\text{g/g}$ body weight, 1 $\mu\text{g}/\mu\text{l}$ 20E). Nerve cords from these abdomens were dissected 5 days postinjection to allow time for migration and fusion of ganglia.

Preadult rise control preparations consisted of P0 ligated abdomens that received physiological saline only (shams) and ligated abdomens receiving no other treatment. When these preparations were sacrificed after 72 hours, the numbers of TM-MFas II-ir fiber tracts in isolated abdomens injected with saline (mean = 8.83 ± 0.95 ; $n = 6$) and in those receiving no other treatment were not statistically different (mean 8.0 ± 1.77 , $n = 6$; Student's *t*-test, n.s.).

Actinomycin D and cycloheximide treatments

We tested whether inhibitors of RNA and protein synthesis could block ganglionic migration and fusion and the concomitant up-regulation of TM-MFas II during the preadult 20E rise that initiates nerve cord reorganization. P0 animals received a single 50- μl injection of actinomycin D (RNA synthesis inhibitor; Sigma Aldrich; A4262) or cycloheximide (a protein synthesis inhibitor; Sigma Aldrich; C6255) or saline vehicle (sham control) into the dorsal thorax or anterior portion of the head. Substances to be injected were dissolved in insect saline (Ephrussi and Beadle, 1936) and filter sterilized (0.22- μm Millipore Ultrafree; Millipore Corp., Billerica, MA). Standard treatments were 100 μg actinomycin D or 200 μg cycloheximide. Similar protocols have been used to block 20E-mediated neuronal death in *Manduca sexta* (Fahrbach et al., 1994; Ewer et al., 1998). Treatment mortality was approximately 20%, similar to that previously reported (Fahrbach et al., 1994). Only healthy animals that showed coordinated motor responses were included in this analysis. Animals treated with inhibitors were slightly less responsive to tactile stimulation than age-matched controls.

Statistical analysis

Means from different treatments in a given experiment were compared with either a two-sample Student's *t*-test (Microsoft Excel; Microsoft Inc., Redmond, WA) or a one-way ANOVA using procedures in R (R Development Core Team, Vienna, Austria; <http://www.R-project.org>). For both kinds of analyses, normality of residuals was examined with Q-Q plots, and variances among three or more groups were compared with Levene's test. When an ANOVA indicated that group means were different, individual contrasts between selected groups were subsequently analyzed using the Student's *t*-test with Bonferroni correction for multiple comparisons to maintain experimentwise error rate at $\alpha = 0.05$.

Dunnett's test (Dunnett, 1964) was used to compare multiple experimental groups with a common control group (Figs. 4, 7) and was calculated in Systat (Systat Software Inc., San Jose, CA). All statistical tests were two-tailed, and *P* above 0.05 or Bonferroni-adjusted values were deemed not significant (n.s.).

RESULTS

Overview of Fas II expression in the ventral nerve cord of *Manduca sexta*

Within the ventral nerve cord of *Manduca sexta*, fasciclin II immunoreactivity was found, as predicted, primarily on the surface of neurons, axons, and glial cells. The two antifasciclin II antisera, one specific for TM-MFas II and the other specific for GPI-MFas II, produced distinct patterns of immunolabeling. Throughout the postembryonic life of the moth, GPI-MFas II was expressed in a stable pattern on the surface of all ganglia and on the transverse nerve associated with each ganglion. TM-MFas II, however, displayed a dual expression pattern within the ventral nerve cord. Very small numbers of TM-MFas II fibers were consistently immunolabeled from the fourth larval instar through the second day of adult life, but the period of ganglionic migration and fusion was characterized by extensive additional immunolabeling of numerous fibers and neuronal somata, particularly in migrating ganglia. TM-MFas II was expressed by diverse neuronal populations: motoneurons, neurosecretory neurons, and intermediate-sized interneurons with intersegmental projections. TM-MFas II was expressed on both somata and axons. TM-MFas II was largely absent from the imaginal nest cells, which are a collection of postembryonically derived local interneurons that are easily identified by their tightly clustered appearance and stereotypical positions (see Fig. 2 of Booker and Truman, 1987). TM-MFas II immunoreactivity was not associated with any population of glial cells.

Temporal profile of TM-MFas II expression in the ventral nerve cord

During formation of the pterothoracic ganglion, T3 fuses first with the next most posterior ganglion, the first abdominal ganglion (A1), and then with the next most anterior ganglion, T2. This process involves the condensation of more than 1 mm of interganglionic connective during the first 3 days following pupal ecdysis (Amos et al., 1996). T3 thus serves as an example of a ganglion that migrates a relatively large distance and fuses at both its posterior and its anterior margins.

In the fourth larval instar, prior to the commencement of ganglionic migration, only a single pair of longitudinal TM-MFas II-ir fibers was visible in the CNS. This pair of fibers extended the entire length of ventral nerve cord (Fig. 1A). Several additional TM-MFas II-ir fibers were observed in feeding fifth-instar larvae; at this time, two prominent neurons were tentatively identified as the ventral unpaired median (VUM) cells on the basis of their unique morphology (Fig. 1B). Expression of TM-MFas II was more extensive on the first day of wandering (W0), with additional TM-MFas II-ir fibers and labeled neuronal somata now evident (Fig. 1C). At this time, a cortical rind of punctate TM-MFas II immunoreactivity was also observed (Fig. 1C, inset). After the fifth instar had molted to the pupal stage, 10–12 fibers per ganglion exhibited TM-MFas II immunoreactivity, and the number of immunolabeled neuronal somata was markedly increased (Fig. 1D). On P1, the number of TM-MFas II-ir fibers visible in T3 increased even further, with 13–15 fibers per ganglion now clearly visible (Fig. 1E). TM-MFas II labeling of fibers in the ventral nerve cord was sustained at this level through stage P2 (Fig. 1F). On P3, the number of TM-MFas II-ir fibers reached a peak, with 19–21 immunolabeled fibers per ganglion. At this time, there was a reduction in TM-MFas II expression on neuronal somata (Fig. 1G). The initial, highly restricted pattern of a single pair of TM-MFas II-ir longitudinal fiber tracts was again observed in the pharate adult, by which time the formation of compound ganglia was

complete (Fig. 1H). Two days after adult ecdysis, these two longitudinal tracts were no longer immunolabeled (Fig. 1I). Thus, with the exception of a pair of longitudinal fibers, expression of TM-MFas II in T3 was temporally correlated with ganglionic migration and fusion.

We next asked whether modulation of TM-MFas II immunoreactivity is restricted to ganglia that undergo migration. The third, fourth, and fifth abdominal ganglia (A3, A4, and A5) provide examples of nonmigrating ganglia. Abdominal ganglia that do not migrate had far fewer TM-MFas II-ir fibers and somata than migrating ganglia (Fig. 2A). A direct comparison of TM-MFas II labeling in representative T3 and A5 ganglia on P2 is presented in Figure 2B,C. In T3, approximately 16 fiber tracts were TM-MFas II-ir, and numerous neuronal somata were immunolabeled; in A5, only four fibers and fewer TM-MFas II-ir somata were observed.

The difference in the number of TM-MFas II-ir fibers in migrating and nonmigrating ganglia is shown in Figure 3, which presents counts of TM-MFas II-ir fibers in the T3–A1 (dark bars) and A4–A5 (light bars) interganglionic connectives. Figure 3A and the overlay in Figure 3B provide a schematic representation of the three separate episodes of 20E secretion that drive metamorphosis: the commitment pulse, the prepupal peak, and the preadult rise. Note that the preadult rise in 20E, which is required for migration and fusion, is associated with a significant increase in the number of TM-MFas II-ir fibers. The earlier prepupal peak, required to prepare the CNS to respond maximally to the preadult rise (Amos et al., 1996), is temporally correlated with a smaller increase in the number of TM-MFas II-ir fibers.

Fas II expression in the ventral nerve cord following removal of 20E

In the absence of 20E (as a result of ligation on P0), all ganglia remained fixed in their larval locations, which is consistent with previous reports (Amos et al., 1996). The typical increase in expression of TM-MFas II that normally accompanies the start of adult development also did not occur. In fact, a progressive decline in TM-MFas II immunoreactivity was observed over time in abdomens from pupae ligated on P0. TM-MFas II labeling is compared in T3 from a control P0 pupa (Fig. 4A) and a pupa ligated on P0 and sacrificed 9 days later (Fig. 4B). In long-survival ligated abdomens, which were healthy and responsive to tactile stimulation, only sparse immunolabeled longitudinal fiber tracts were visible.

The number of TM-MFas II-ir fiber tracts in the T3–A1 connectives was compared in control and ligated P0 pupae (Fig. 4C). Control connectives contained 12.0 ± 0.6 (mean $\pm$ SEM) TM-MFas II-ir fibers on P0. As early as 3 days after ligation, the number of TM-MFas II-ir fibers was significantly different from that in nonligated (intact) controls (8.0 ± 0.5 ; Dunnett's test, $P < 0.005$) and lower than that in normal animals at stage P3 (see Fig. 3B). The number of TM-MFas II-ir fibers in the T3–A1 connectives of abdomens ligated on P0 continued to decrease over the period following ligation, with 3.2 ± 1.1 and 3.0 ± 0.6 immunolabeled fiber tracts present when 20E was absent for 9 and 10 days, respectively. This finding suggests that 20E is needed not only for the increase in the number of TM-MFas II fiber tracts but also for the maintenance of its expression.

One possible nonendocrine explanation of the ligation-related reduction in number of TM-MFas II fibers might be the loss of descending long-distance projecting neuronal fibers as a result of neuronal death caused by ligation. This explanation of our results is likely incorrect, because ligated animals can avoid or reverse reduction in the number of TM-MFas II fibers if exogenous 20E is provided (see supporting data below). To address this possibility directly, however, we transected connectives between the T2 and the T3 ganglia in late fifth-instar larvae and counted TM-MFasII-ir fibers in the ventral nerve cord between T3 and A1. Because complete transection resulted in aberrant ganglionic migration (data not

shown), we instead analyzed the effects of cutting one of the two paired connectives. In pupae with a transected connective killed at stage P3, the mean number of TM-MFas II fiber tracts in both left and right T3–A1 connectives was 20.17 ± 1.1 ($n = 6$), which was not statistically different (Student's t -test) from the number of fibers counted in control P3 animals (mean 20.00 ± 0.31 , $n = 5$). These data indicate definitively that removal of descending interneurons by neuronal death subsequent to ligation does not contribute significantly to the decline of TM-MFas II labeling in the ligated animals.

Response to 20E replacement

The nature of the signal missing in ligated abdomens can be inferred from hormone-replacement experiments. The profile of TM-MFas II immunoreactivity in a larva ligated on W3 (after experiencing the prepupal peak but before the preadult rise) is shown in Figure 5. Approximately five TM-MFas II-ir fiber tracts were observed in the T3–A1 connectives (Fig. 5A); three or four TM-MFas II-ir fiber tracts were visible in A2 (Fig. 5B). In contrast, ligated abdomens that received an infusion of 20E contained significantly more TM-MFas II-ir fiber tracts (Fig. 5C,D). In addition, larger numbers of neuronal somata were TM-MFas II-ir in abdomens receiving 20E replacements. In conjunction with the increased expression of TM-MFas II, animals receiving 20E replacement also displayed striking decreases in the distances of T3 through A2 (Fig. 5C,E). Other migration-related events were also apparent, including development of the “ghost” of A2. The number of TM-MFas II-ir fibers was significantly different (Student's t -test, $P < 0.01$) between the 20E-infused ($n = 4$) and control pupae ($n = 5$), and interganglionic distances between T3 and A1 were significantly shorter between infused and noninfused controls (Student's t -test, $P < 0.01$).

These results, based on the removal and replacement of the preadult rise of 20E, implicate this endocrine event in the regulation of TM-MFas II expression in the ventral nerve cord of *Manduca sexta*. We used ligation and hormone replacement to investigate further the role of the earlier prepupal peak of 20E in the regulation of TM-MFas II expression in the ventral nerve cord. Ganglia T3 and A1 from larvae ligated on W0 and therefore not exposed to the prepupal peak of 20E displayed few longitudinal fibers and faintly labeled somata (Fig. 6A,B). Control larvae sampled on W3 that had experienced the endogenous prepupal peak of 20E contained more TM-MFas II-ir fibers in the T3–A1 connectives, plus TM-MFas II-ir neuronal processes with a morphology characteristic of the VUM neurons (Fig. 6C; Pflüger et al., 1993; Lehman et al., 2000). T3 and A1 ganglia from larvae that were ligated on W0 and subsequently received an infusion of 20E that mimicked the prepupal peak, as predicted, showed immunolabeling comparable to that observed in control animals (Fig. 6E,F).

Fewer TM-MFas II-ir fibers were counted in ganglia lacking 20E (W0-ligated animals, $n = 4$) than in ganglia that experienced the natural 20E peak (nonmanipulated W3 larvae, $n = 9$; Student's t -test with the Bonferroni correction, $P < 0.017$) or those preparations that had their 20E artificially replaced ($n = 4$; Student's t -test, $P < 0.01$). The Bonferroni correction lowered the critical significance level from 0.05 (α) to 0.017 (i.e., $\alpha/3$ experimental comparisons). There was no difference in the number of TM-MFas II-ir fibers in the 20E prepupal peak-replacement samples compared with W3 control preparations (Student's t -test, n.s.).

Treatment of intact insects with inhibitors of RNA and protein synthesis during the preadult 20E rise

In this series of experiments, we aimed to provide additional support for the conclusion that 20E regulates nerve cord reorganization by regulating the expression of TM-MFas II. Because the steroid 20E is a well-known regulator of transcription in arthropods (Riddiford et al., 2000), we argued that, if 20E has a direct action on TM-MFas II production, then this

regulation would be susceptible to inhibitors of RNA or protein synthesis; furthermore, this predicted loss of TM-MFas II expression would be matched with a concomitant block of ganglionic migration and fusion. By injecting the protein synthesis blocker cycloheximide on day P0 and sacrificing the insects 4–5 days later, we determined that this inhibitor prevented migration and fusion and the increase in TM-MFas II labeling in 100% of insects examined ($n = 10$; Fig. 7). We also injected animals at stage P0 with the transcription blocker actinomycin D and, after waiting 4–5 days, observed a similar blockade of events in 100% of healthy animals tested ($n = 5$). With both inhibitor treatments, nerve cords showed no signs of ganglionic migration and fusion and strikingly resembled the nerve cords of P0 animals prior to changes induced by the 20E preadult rise (Amos and Mesce, 1994; Amos et al., 1996).

Steroidogenesis in insect prothoracic glands is stimulated by prothoracicotropic hormone (PTTH) through a cascade of transcription- and translation-independent events, including Ca^{2+} influx, an increase in cAMP, and phosphorylation of ribosomal protein S6 (Gilbert et al., 1997). PTTH-induced steroidogenesis, however, is reduced (but not blocked) in the presence of cycloheximide and actinomycin D (Keightley et al., 1990). To ensure that the effect of the inhibitors on TM-MFas II expression in the connectives reflected actions on the ventral nerve cord rather than on the prothoracic gland, we treated additional animals with combinations of 20E and cycloheximide or actinomycin D. The results of studies with inhibitor-20E cocktails were not different from those in which the inhibitors were administered without additional steroid (Fig. 7).

Evidence for fasciculation during ganglionic migration

Morphological evidence suggesting the importance of fasciculation during migration and fusion is found in TM-MFas II labeling patterns apparent within ganglia during the peak of their migration. For example, when migration events for T3 were most prominent at stages P2 and P3, primary neurites and numerous longitudinal fascicles became intensely TM-MFas II immunoreactive (Fig. 8A), and numerous labeled somata appeared to be tethered via their primary neurites to TM-MFasII-ir longitudinal fiber tracts (Fig. 8B). This pattern of immunolabeling was observed within the neuropils of all migrating ganglia.

Expression of GPI-MFas II during postembryonic reorganization of the CNS

In contrast to the spatial and temporal profiles reported here for the TM-MFas II isoform, GPI-MFas II expression was not modulated in the ventral nerve cord during metamorphosis. All ganglia expressed GPI-MFas II (Fig. 9). The GPI-MFas II immunolabeling appeared to be confined to the surface of the ganglia and took the form of intercalating circles, giving the impression of an organized cuboidal epithelium (Figs. 8C, 9B); this pattern likely represents the immunolabeling of the “surface” category of glial cells previously described (Freeman and Doherty, 2006). GPI-MFas II expression was most robust on the transverse nerve (Fig. 9A,D). This immunolabeling may represent GPI-MFas II-ir glial cells within the transverse nerve, insofar as glial cells have been previously shown to be a component of this nerve (Wall and Taghert, 1991; Hesterlee and Morton, 2000). Transverse nerves were labeled with equal intensity in migrating and nonmigrating ganglia. GPI-MFas II-ir was also present on the web-like vacant sheath (i.e., the “ghost”) created by the anterior migration of A2 (Fig. 9D). GPI-MFas II labeling was also observed on the transverse nerve and nerve roots of A2, which remained associated with the A2 “ghost” (Fig. 9D).

20E-independent expression of TM-MFas II

Our observations show that important elements of TM-MFas II expression depend on 20E titers. However, a pair of uninterrupted TM-MFas II-ir fiber tracts, visible both in the segmental ganglia and in the interganglionic connectives, was observed even when 20E

titers were low. The fact that this pair of TM-MFas II-ir fiber tracts spanned the entire length of the ventral nerve cord suggested that they might be the processes of the VM neurosecretory neurons of the brain. The four VM neurons have an unusual, possibly unique cytoarchitecture. They consist of two bilaterally symmetrical pairs of somata in the ventromedial brain. The tightly fasciculated VM axons travel ipsilaterally through the CNS (appearing as one fiber per connective) and project into the proctodeal nerve to a neurohemal organ (Truman and Copenhaver, 1989). The neuropeptide EH is released from the proctodeal nerve in the larva and the pupa to coordinate ecdysis: during adult development, the VM cells also project collateral axons to the corpora cardiaca, forming a new release site used for adult ecdysis (Truman and Copenhaver, 1989; Hewes and Truman, 1991; Riddiford et al., 1994).

Colocalization of TM-MFas II and EH immunoreactivity on this set of medial fibers in T2 of a fourth-instar larva is shown in Figure 10. At P0, multiple fibers per ganglion displayed TM-MFas II labeling, but only the most medial pair of longitudinal fibers was both TM-MFas II-ir and EH-ir (Fig. 10B). Double labeling was also present on a single pair of longitudinal fibers at the end of adult development, when other TM-MFas II immunolabeling was absent (Fig. 10C). TM-MFas II immunoreactivity was undetectable 2 days after adult ecdysis (Fig. 10D). These longitudinal fibers, however, continued to show EH immunoreactivity 5 days post-adult ecdysis (Fig. 10D).

Brains were immunolabeled to confirm that the colocalized TM-MFas II and EH medial fiber tracts connected to the paired EH-positive somata visible in the ventromedial region of the brain. As expected, four TM-MFas II-ir VM neurons were visible at the midline of the ventral brain (Fig. 11A). The VM neurons were unambiguously continuous with fibers in the connectives linking the brain to the subesophageal ganglion (SEG; Fig. 11B). Colocalization of TM-MFas II and EH labeling was apparent in the VM somata and their posterior-projecting axons (Figs. 10B, 11A). A continuous pattern of coexpression was observed in these axons throughout all segmental ganglia of the ventral nerve cord (data not shown).

Because the two pairs of VM neurons have been described previously as the only CNS neurons containing EH (Riddiford et al., 1994), we were surprised to find that the EH antiserum also labeled an additional population of five somata located in the dorsolateral region of the protocerebrum (Fig. 11A). These dorsolateral neurons also expressed TM-MFas II (Fig. 11A). The neurons appear to be type Ia₂ neurosecretory neurons. The processes of these neurons projected to the CC-CA complex (Fig. 11C). Both somata of the EH (i.e., VM) and EH-like (i.e., type Ia₂) neurons expressed TM-MFas II until the second day of adult life, after which TM-MFas II labeling disappeared.

DISCUSSION

Despite the pervasiveness of ganglionic migration and fusion among the Arthropoda and other invertebrate phyla (Bullock and Horridge, 1965), the cellular mechanisms underlying this remarkable event have rarely been studied (Amos and Mesce, 1994). We examined the expression of insect CAMs known to play significant roles in the assembly of the nervous system during embryogenesis and therefore potentially available to be used again during postembryonic development (Wright et al., 1999; Wright and Copenhaver, 2000, 2001). We have established that TM-MFas II expression is up-regulated immediately preceding and during the time of ganglionic migration. We have also shown that the expression of TM-MFas II in the ventral nerve cord is dependent on exposure of the ventral nerve cord to 20E and that the effects of 20E treatment are blocked by cycloheximide and actinomycin D. These observations are consistent with our hypothesis that steroid-regulated expression of TM-MFas II is required for ganglionic migration and fusion.

Overview of Fas II expression during development of the CNS

Wright and Copenhaver (2001) examined the activity profile of the transmembrane and GPI-linked forms of fasciclin II in the CNS and the enteric nervous system (ENS) during embryogenesis in *Manduca sexta*. From 22% to 30% of embryogenesis, transient TM-MFas II-ir was observed in the CNS (abdominal ganglia) on commissural fibers. Later, between 30% and 80% of embryonic development, up to 16 separate longitudinal fiber tracts were TM-MFas II-ir, with maximal expression observed at 60% of development (Wright and Copenhaver, 2001). Subsets of neuronal somata also became TM-MFas II-ir at 60% of development. The immunolabeled somata included the previously described VUM octopaminergic neurons (Pflüger et al., 1993; Lehman et al., 2000; Mesce, 2002). TM-MFas II expression in migrating ganglia at the larval–pupal transition (this report) therefore mirrors the dynamic expression profile observed during embryogenesis. Wright and Copenhaver (2001) reported that TM-MFas II is rapidly trafficked to elongating regions of axons; as a result, somata are only briefly TM-MFas II-ir. This observation may explain why, in the present study, the VUM neurons in fifth-instar larvae initially showed intense label that waned prior to the appearance of intensely labeled longitudinal fiber tracts.

After hatching, TM-MFas II expression disappeared almost completely from the ventral nerve cord (Wright and Copenhaver, 2001). Similarly, TM-MFas II labeling was largely absent from the adult CNS in our studies. This suggests that the adult stage is a time of morphological stability, which is in accordance with past observations of the adult CNS in this short-lived moth.

Although TM-MFas II immunolabeling was largely absent in hatchlings, it reappeared transiently on the VUM neurons during the first 2 days of the first larval instar (Wright and Copenhaver, 2001). The VUM cells modulate activity in the larval leg muscles (Consoulas et al., 1999). We observed that, during subsequent larval stages, the VUM cells showed no TM-MFas II immunoreactivity, but, by the early fifth instar, TM-MFas II was once again expressed by the VUM neurons. Knittel et al. (2001) have suggested that persistent expression of TM-MFas II on the surface of the VUM neurons reflects the continuous axonal elongation required to accommodate growth of their muscle targets.

Wright and Copenhaver (2001) also reported the appearance of TM-MFas II immunolabeling on the transverse nerve at between 45% and 80% of embryogenesis. At the late larval, pupal, and adult stages that we examined, only GPI-MFas II was expressed on the surface of the transverse nerve. Tightly fasciculated motor and neurosecretory fibers project through the transverse nerve, ensheathed by glial cells (Wall and Taghert, 1991; Hesterlee and Morton, 2000), and these glial cells may be the source of the GPI-MFas II labeling.

Analysis of the developing ENS has also provided information about the functions of MFas II (Wright et al., 1999; Wright and Copenhaver, 2000; Copenhaver, 2007). In the embryo, a group of 300 enteric plexus (EP) neurons migrates along the fore- and midgut, extending axons along longitudinal muscle bands surrounding the gut (Copenhaver and Taghert, 1989). A population of glial precursor cells then spreads along the same pathways utilized by the EP cells and subsequently ensheathes the neurons (Copenhaver, 1993). TM-MFas II was expressed by EP neurons, beginning at 43% of embryogenesis, whereas tightly adhering or slow-moving immature neurons and glial cells were instead GPI-MFas II-ir (Wright and Copenhaver, 2000). On the basis of these studies of the developing ENS, it has been proposed that TM-MFas II promotes cell motility and axon fasciculation, whereas GPI-MFas II acts primarily as an adhesive substrate for growing neurons (Wright and Copenhaver, 2000, 2001).

Similarly, in the present study, we observed that expression of TM-MFas II was strongly correlated with the dynamic events of ganglionic migration. In particular, numerous TM-MFas II-ir somata and fiber tracts appeared in ganglia that moved, and expression increased as ganglionic migration and fusion progressed. The two most dynamic periods in the life of the ventral nerve cord—embryogenesis and metamorphosis—are accompanied by the up-regulated expression of TM-MFas II. By contrast, GPI-MFas II expression is associated with more stable neural structures, and its expression is not up-regulated during embryogenesis and metamorphosis. Based on the many specimens we examined, we saw no evidence that the GPI isoform of MFas II was present on neurons, in contrast to the TM isoform, which was almost exclusively neuronal. GPI-MFas II expression on glial cells may help to provide structural integrity to prevent the potential collapse of the ganglion. For example, Fushima and Tsujimura (2007) found that *Drosophila* mutants lacking Fas II exhibited a structural collapse of the lobes comprising the mushroom bodies of the brain during metamorphosis.

Why is Fas II expressed on axons during reorganization of the ventral nerve cord?

As its name implies, fasciclin was identified on the basis of its ability to mediate fasciculation of growing axons into bundles. Our data support a role of TM-MFas II in fasciculation. TM-MFas II is also important for mediating axonal elongation, which is an essential feature of ventral nerve cord reorganization in *Manduca sexta* (Amos and Mesce, 1994). Axonal elongation is best understood by focusing on motoneurons in the pterothoracic ganglion. The somata of the monopolar motoneurons in A1 and A2, for example, move anteriorly into the forming pterothoracic ganglion. The axon terminals of motoneurons, however, remain in persistent, close contact with their muscle targets, even if the muscle undergoes remodeling during metamorphosis (Weeks and Truman, 1986; for review see Consoulas et al., 2000; Knittel and Kent, 2002, 2005). These constraints present a puzzle: How does an axon grow over a long distance when its soma and axon are anchored at both ends? The most parsimonious explanation of such growth is consistent with the idea that axons elongate via a process referred to as *interstitial* axon growth (Rossi et al., 2007). Little is known about the underlying cellular mechanisms mediating this process, but interstitial growth is likely to be a widespread feature of CNS development in both vertebrates and invertebrates. For example, in the rat brain, interstitial axon elongation is characteristic of Purkinje cells, which synapse on their targets before the major phase of cerebellar growth (Sotelo, 2004).

In our scenario, TM-MFas II is envisioned as being transported from the soma to proximal regions of the axon. As the axons of motoneurons elongate between their somata and their original nerve root, which remains relatively fixed, TM-MFas II may be continuously added to the newly produced axonal membrane. This is a fundamentally different process from the growth-cone-mediated extension at the axon terminal (for review see Goodman et al., 1997). Adhesive interactions between adjacent axons in the neuropil or at the fixed nerve roots (homo- and/or heterophilic interactions) may provide the necessary anchoring force to signal and thus promote interstitial growth. It remains unknown whether the process of rapid (1 cm/day) interstitial axon elongation observed during ventral nerve cord reorganization simply accommodates ganglionic migration or more actively propels neurons to their new locations, thus contributing to the motile forces underlying ganglionic migration.

Despite the present focus on ganglionic migration and fusion, we want to state clearly that the up-regulation of TM-MFas II expression is very unlikely to be solely tied to the process of ganglionic migration, because numerous cellular events occur in the ventral nerve cord of *Manduca sexta* during metamorphosis (Fahrbach et al., 2001; Fahrbach and Weeks, 2002). Some of these events, however, are highly unlikely to be associated with the large-scale up-regulation of TM-MFas II that we observed on the axons of neurons in migrating ganglia. For example, the large increase in the number of TM-MFas II-ir fibers in the connectives

cannot be attributed to the differentiation and growth of new neurons (i.e., imaginal nest cells), because such cells are almost exclusively local interneurons shown not to project out the nerve roots or connectives (Booker and Truman, 1987). Dendritic and neuritic pruning is also not a major factor, insofar as the consolidation of multiple migrating neurons ultimately results in a greater overall volume that houses a given translocated neuron. For example, neurons in A1 or A2 span multiple neuromeres within their newly formed pterothoracic ganglion (see Fig. 4 of Mesce and Fahrbach, 2002). Also, at the end of metamorphic development, the total length of the nerve cord, despite its global reorganization and ganglionic consolidation, is about the same as in the last-instar larva (Amos and Mesce, 1994; Amos et al., 1996). We do not yet know whether the dendritic processes of translocated neurons up-regulate TM-MFas II expression, but this change would presumably occur later, at approximately stage P7, when ganglionic fusion is near completion and new synaptic connections are forming within the pterothoracic ganglion (Amos and Mesce, 1994). This temporal disparity indicates that this type of remodeling cannot account for the robust up-regulation that we observed during the first few days of pupal–adult development.

Another caveat is that TM-MFas II is unlikely to be the only CAM involved in ventral nerve cord reorganization. Neural development in general appears to require multiple and redundant CAM function (Forni et al., 2004). Genetic analysis using *Drosophila* loss-of-function mutations in genes encoding CAMs such as neuroglian (homolog of vertebrate L1) and fasciclin have resulted in extremely subtle defects in axon guidance and fasciculation, consistent with overlapping CAM functions (Goodman et al., 1997). Double knockouts are typically necessary to unmask the function of CAMs. For example, CNS axon patterning is disrupted much more severely in *neurotactin* and *neuroglian* *Drosophila* double mutants than in mutants lacking only one of these CAMs (Speicher et al., 1998).

20E regulation of ventral nerve cord reorganization

Although it is well-established that ecdysteroids regulate neurometamorphosis in insects (Fahrbach et al., 2001; Fahrbach and Weeks, 2002), this study is the first to demonstrate that TM-MFas II expression is dependent on the presence of ecdysteroids. Our report also complements previous studies showing that ganglionic migration and fusion are governed by the ecdysteroids (Pipa, 1969; Amos et al., 1996), extending what is known about potential downstream events regulating nerve cord change.

Treatment with inhibitors of RNA and protein synthesis blocked the increase in TM-MFas II expression observed during pupal–adult development. This loss was also associated with the prevention of ganglionic migration and fusion, providing strong supporting evidence that 20E acts on target cells to affect gene regulation and the synthesis of proteins involved in nerve cord reorganization. A similar dependence was found for the 20E-mediated active cell death of motoneurons that occurs at the end of larval life and at the end of adult development (Fahrbach et al., 1994; Hoffman and Weeks, 1998).

Steroid-regulated nerve cord reorganization during metamorphosis provides a promising system for study of how circulating hormones choreograph segment-specific developmental events. One factor regulating segment-specific development is likely to be segment- and cell-specific expression of ecdysone receptors (EcR), the nuclear receptors that mediate the cellular effects of ecdysteroids (Riddiford et al., 2000). Relatively few studies have documented the cell-by-cell and ganglion-specific pattern of isoform-specific EcR expression in the CNS of *Manduca sexta* (Fahrbach and Truman, 1989; Bidmon et al., 1991a,b; Fahrbach, 1992; Fahrbach et al., 2001). Although these previous studies indicate that expression of EcR is widespread in neurons and glial cells in the CNS at the larval–pupal transition, direct comparisons of EcR expression in migrating and nonmigrating ganglia have not been made.

No prior studies in insects have demonstrated 20E regulation of CAM expression. Several studies in vertebrates, however, have demonstrated a link between gonadal steroids and CAM expression. For example, in female rats, the steroid hormone fluctuations of the ovarian cycle alter expression of PSA-NCAM (the polysialylated form of the neural cell adhesion molecule) in gonadotropin-releasing hormone neurons undergoing synaptic remodeling (Parkash and Kaur, 2005).

Constitutive expression of MFas II

Two populations of neurons chronically expressed TM-MFas II during postembryonic life: the EH-containing VM neurons and the type Ia₂ lateral neurosecretory neurons. Both populations of neurons have tightly fasciculated axons and were immunoreactive to an EH antiserum. The somata of the four VM neurons are located in the brain but project to neurohemal release sites in the segmental ganglia and the proctodeal nerve. In larvae, the axons of the VM neurons must accommodate a massive increase in body size during larval development by continuously adjusting their axonal length (Truman and Copenhaver, 1989; Hewes and Truman, 1991). The VM neurons may therefore utilize TM-MFas II for interstitial growth of their axons. By contrast, the tightly fasciculated axons of the five pairs of type Ia₂ neurons project only a relatively short distance to the CC-CA complex in the head. Possibly, these cells retain TM-MFas II to remain fasciculated to accommodate specific cytoarchitectural changes during metamorphosis (Nijhout, 1975). This is not a particularly compelling explanation, in that there are nearly 65 other neurosecretory neurons in the brain that share this projection pattern (Homberg et al., 1991), and they do not express TM-MFas II. The VM and EH-like-lateral cells possibly share another, as yet unknown, functional or lineage-based feature.

GPI-MFas II expression on the ventral nerve cord was not observed to be temporally or spatially regulated during the larval–pupal transition, consistent with previous reports (Higgins et al., 2002). The GPI-MFas II we observed was apparently expressed on the surface of glial cells, also consistent with previous reports (Cantera and Trujillo-Cenoz, 1996; Wright and Copenhaver, 2000, 2001; Higgins et al., 2002). The lack of glial-specific markers precludes a definitive categorization of the cell types expressing GPI-MFas II. We suggest, based on the descriptions and nomenclature of glial cell types presented by Freeman and Doherty (2006), that the surface glia of the ganglion and, perhaps, the neuropil glia, are the cell types that were immunolabeled.

CONCLUSIONS

The process of ventral nerve cord reorganization provides a rich biological system for exploration of the role of insect CAMs in metamorphosis. The spatially and temporally restricted TM-MFas II expression patterns suggest that modulation of cell adhesion is a key mechanism of ganglionic migration and fusion and, possibly, interstitial axon elongation. The timing of this modulation is controlled by pulses of ecdysteroids, which regulate the transcription of TM-MFas II. Our data showing that GPI-MFas II expression is not regulated by similar metamorphic changes in 20E are consistent with the hypothesis that the GPI isoform of MFas II is associated with structures (e.g., nerve roots or ganglionic sheaths) that require stabilization by glial cell scaffolds during metamorphosis.

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Fig. 1.

Dynamic expression of the transmembrane isoform of *Manduca sexta* fasciclin II (TM-MFas II) during metamorphosis of the ventral nerve cord. Three diagrams of the ventral nerve cord positioned to the left and right of the photomicrographs provide a guide to the location of migrating ganglia. The “ghost” of either the first (A1) or second (A2) abdominal ganglion refers to the vacant sheath left behind after ganglionic migration. Sample sizes indicate the number of specimens examined at each stage of development. **A:** TM-MFas II immunoreactivity in a fourth-instar larva prior to the onset of ventral nerve cord reorganization. Only one pair of longitudinal fibers is visible in the third thoracic ganglion (T3; $n = 8$). **B:** In an early fifth-instar larva, additional TM-MFas II-immunoreactive (ir) fibers are visible, and a pair of large midline neurons is also TM-MFas II-ir ($n = 9$). **C:** On

W0, the number of TM-MFas II-ir fibers has increased ($n = 9$). The pair of TM-MFas II-ir midline neurons and their associated processes are reminiscent of the ventral unpaired median (VUM) neurons (straight arrow). A diffuse and punctate pattern of TM-MFas II labeling is also observed at the perimeter of the ganglion (arrow-head). **Inset:** The cortical rind of punctuate TM-MFas II labeling (arrowhead) and TM-MFas II labeling around commissural tracts (curved arrow) appears brightest at W2, shown here. **D:** At P0, 10–12 fibers per ganglion exhibit TM-MFas II immunoreactivity ($n = 14$). Labeled TM-MFas II-ir somata are more numerous and are now visible in all regions of the ganglion. **E:** At stage P1, the number of TM-MFas II-ir fibers increases as ganglionic migration proceeds, with 13–15 fibers per ganglion now labeled ($n = 12$). Note the presence of immunolabeled fibers in the lateral nerves (arrowhead). **F:** On P2, 15–17 TM-MFas II-ir fibers per ganglion are observed ($n = 9$). **G:** On P3, the first abdominal ganglion (A1) has fused with T3. At this point, TM-MFas II expression reaches its peak, with 17–19 fibers per ganglion labeled ($n = 5$) and a concomitant loss of staining on somata. **H:** About 2 weeks later, TM-MFas II expression reverts to the pattern observed prior to the onset of ventral nerve cord reorganization. Only one set of longitudinal fibers per ganglion is visible ($n = 8$), as in A. **I:** On day 2 of adult life (day +2), TM-MFas II expression is absent on all fibers and somata ($n = 9$). A1–6, abdominal ganglia 1–6; SEG, subesophageal ganglion; T1–3, thoracic ganglia 1–3; TAG, terminal abdominal ganglion. Scale bar = 100 μm .

Fig. 2.

TM-MFas II expression is up-regulated in ganglia that migrate and are in the process of fusing, but not in nonmigrating ganglia. The diagram of the ventral nerve cord at left indicates the relative positions of ganglia that migrate, which include the SEG through A2, and A6. All these ganglia will eventually fuse with at least one neighboring ganglion, except for T1, which shows significant migration but does not fuse with adjacent ganglia (Amos and Mesce, 1994; Cantera et al., 1995). **A:** Immunolabeling of the entire CNS with an antibody specific for TM-MFas II is shown at stage P2. Ganglia not involved in the formation of the pterothoracic ganglion (A3–A5) show low-intensity immunolabeling. The

ganglion-shaped structure below A2 is the “ghost” of the cortical structure that formerly surrounded the neurons and neuropil of A2. **B,C:** Higher resolution confocal images of TM-MFas II expression in a migrating ganglion (T3; B), and in a nonmigrating ganglion (A5; C) at stage P2. The number of stained fibers was counted in the horizontal plane delineated by the two arrows. In the connectives posterior to T3, approximately 16 fiber tracts are TM-MFas II-ir (not including fibers exiting the CNS through lateral nerve roots); in contrast, in A5, only four intensely stained fiber tracts are TM-MFas II-ir. A6, which will fuse with the larval TAG to form the adult TAG, migrates later than T3, A1, and A2 and so has a later onset of TM-MFas II immunoreactivity (data not shown). SEG, subesophageal ganglion; TAG, terminal abdominal ganglion. Scale bars = 175 μm in A; 75 μm in C (applies to B,C).

Fig. 3. Developmental profile of the number of TM-MFas II-ir fibers in the connectives displayed across time and space (B). **A:** Relative profile of 20E titers during metamorphosis of *Manduca sexta*, with identification of individual peaks. **B:** Histograms display counts of the numbers of TM-MFas II-ir fibers in the connectives between T3 to A1 and A4 to A5 (see Fig. 2B,C for location of counts). Numbers above bars represent the sample size for each group. The mean for each stage $\pm$ SEM is presented. The number of TM-MFas II-ir fibers in P4–P7 animals was not counted, because ventral nerve cords at this stage were too fragile to withstand immunohistochemical processing. For all histograms shown here, the asterisk denotes a Bonferroni-corrected significance level of $P < 0.0125$ ($\alpha/4$ experimental comparisons). Comparison groups were selected based on the potential influence of 20E fluctuations. The fourth comparison group (W0 vs. W3) was not significant by the Bonferroni-corrected significance level but was significant if experimentwise error was omitted ($P = 0.03$; Student's *t*-test). Statistical comparisons were made between fiber counts in T3–A1; the A4–A5 counts are shown for reference only. The overlay links the counts to

the relative values of the profile of 20E titers during metamorphosis in *Manduca sexta*. (Modified from the Journal of Insect Physiology, vol 42, Amos TM, Gelman DB, Mesce KA, Steroid hormone fluctuations regulate ganglionic fusion during metamorphosis of the moth *Manduca sexta*, p 579–591, Copyright 1996, with permission from Elsevier.)

Fig. 4.

Effect of 20E removal on the number of TM-MFas II-ir fiber tracts (i.e., fascicles) in the ventral nerve cord. Isolated P0 abdomens were sacrificed and ventral nerve cords immunolabeled for TM-MFas II immunoreactivity at 1-day intervals following ligation. In the absence of 20E, ganglia did not migrate ($n = 36$). **A:** Approximately 12 fascicles per ganglion are TM-MFas II-ir in T3 of controls dissected 5 hours postpupation. Note that retracting tracheae, which appear black in the confocal image (arrows), mark the initiation of ganglionic migration. **B:** T3 of an animal ligated on P0 and sacrificed 9 days later. Four fascicles are TM-MFas II-ir, including a pair of longitudinal fiber tracts (arrowheads). In

contrast to the nonmanipulated P0 animal, few somata express TM-MFas II immunoreactivity ($n = 6$). Because ligated preparations remain viable for several weeks (Amos et al., 1996), the loss of labeled fibers is not a consequence of tissue death. **C:** Counts of the number of TM-MFas II-ir fibers in the T3–A1 connective of pupae ligated on P0 and examined after 1-day intervals (up to 10 days). Numbers above bars represent the sample size for each group. The mean for each stage $\pm$ SEM are presented. $*P < 0.01$ by Dunnett's test with the nonmanipulated P0 group serving as the reference value. This significant difference is first achieved 3 days after ligation, as indicated, and increases over the days following ligation (not indicated). Scale bar = 75 μm in A; 50 μm for B.

Fig. 5.

Demonstration that the preadult rise of 20E is necessary and sufficient for the increase of TM-MFas II expression observed during ventral nerve cord reorganization. Larvae were ligated on W3 after experiencing their natural prepupal peak. After pupal ecdysis (P0), animals were treated (injected or infused) with 20E. Pupae were sacrificed after 72 hours of hormone treatment and compared with ligated age-matched controls sacrificed at the time. See Materials and Methods for additional details. Schematic diagrams of the ventral nerve cord outline the positions of ganglia following ligation (A,B) and hormone replacement (C). **A:** TM-MFas II immunoreactivity in the T3–A1 connectives of a W3-ligated animal (no 20E present). Five fiber tracts are TM-MFas II-ir. **B:** Three fiber tracts are TM-MFas II-ir in A2 of the same W3-ligated larva shown in A. **C:** TM-MFas II labeling in the T3–A1 connectives of a W3-ligated larva that received an infusion of 20E. Formation of the pterothoracic ganglion is apparent, as is the “ghost” sheath of A2. **D:** Number of TM-MFas II-ir fiber tracts in the T3–A1 connectives in larvae ligated on W3. The difference in number of TM-MFas II-ir fibers in the T3–A1 connectives was statistically significant (5.8 ± 0.8

fibers compared with 15.5 ± 0.7 ; Student's *t*-test, $*P < 0.01$). Results are expressed as the mean for each age $\pm$ the SEM. Numbers over bars are sample size. **E:** T3–A1 distance after ligation on W3. The interganglionic distance was measured from the center of T3 to the center of A1. In W3-ligated animals, the distance between T3 and A1 was 2.1 ± 0.1 mm, whereas the T3–A1 distance in preadult-rise-infused animals was 0.6 ± 0.2 mm. This difference was statistically significant (Student's *t*-test, $*P < 0.01$). Numbers over bars are sample size. 20E, 20-hydroxyecdysone; Lig, ligated behind the prothoracic segment; TAG, terminal abdominal ganglion. Scale bars = 75 μ m in A; 90 μ m in B; 100 μ m in C.

Fig. 6. TM-MFas II immunolabeling in T3, A1, and their connectives as a consequence of ligation (prior to the prepupal peak) and subsequent 20E replacement. Schematics of ventral cords represent each experimental manipulation. **A,B:** TM-MFas II immunoreactivity in T3 and A1, respectively, of a larva ligated on W0. The medial fiber tracts are TM-MFas II-ir, and few immunolabeled somata are visible. **C,D:** More TM-MFas II-ir fibers and somata are present in T3 and A1 of a larva that experienced the natural prepupal peak of 20E (stage W3). **Inset:** A single optical section showing the TM-MFas II-ir labeled somata that were out of view in D. **E,F:** Expression of TM-MFas II in T3 and A1 of a larva ligated on W0

that subsequently received a replacement of the prepupal peak of 20E; the intensity and pattern of the immunolabeling is similar to that obtained in the control W3 larva (C,D) and greater than in the W0 larva that did not receive the 20E infusion (A,B). **G:** Number of TM-MFas II-ir fibers in the T3 to A1 connective counted in: W0-ligated animals, W3 controls, and W0-ligated animals that received 20E replacement. Compared with W0-ligated animals, there was an 83% increase in the number of labeled fibers in the T3–A1 connectives of ligated animals receiving the prepupal peak 20E infusion (8.25 ± 0.60 compared with 4.50 ± 0.65 ; Bonferroni-corrected Student's *t*-test $*P < 0.003$; $\alpha/3$ experimental comparisons). The number of nerve cords immunolabeled is provided above each bar. No difference was observed in the number of TM-MFas II-ir fibers labeled between 20E replacement and W3 animals ($P > 0.05$). 20E, 20-hydroxyecdysone; Lig, ligated behind the prothoracic segment; TAG, terminal abdominal ganglion. Scale bar = 100 μm .

Fig. 7.

Comparison of the number of TM-MFas II-ir fiber tracts present and the degree of ganglionic migration among insects injected with inhibitors of protein and mRNA synthesis. For tests of statistical significance, the sham group served as the Dunnett's test reference group. **A:** TM-MFas II-ir fiber tracts present in the T3–A1 connectives among insects injected with saline ($n = 7$), the protein synthesis inhibitor cycloheximide ($n = 10$), and the inhibitor of RNA actinomycin D ($n = 5$). All sacrificed animals ($n = 22$) were healthy and examined 4–5 days after injection on day P0. The difference between the numbers of TM-MFas II fascicles in inhibitor-treated and saline-injected animals was statistically significant (Dunnett's test, $*P < 0.0001$). To control for the possibility that inhibitors of translation and transcription limited TM-MFas II expression and ganglionic migration by lowering the concentration of 20E, animals at stage P0 were injected with a cocktail of 20E plus either cycloheximide or actinomycin and examined 4–5 days later. Even in the presence of injected 20E, both inhibitors prevented normal TM-MFas II expression. There was no difference in the counts of TM-MFas II-ir fibers tracts in inhibitor-treated preparations regardless of the presence or absence of 20E (Student's t -test, n.s.). **B:** In 100% of inhibitor-treated insects, signs of ganglionic migration and fusion were absent, but not in saline-injected animals. The distance measured between the midpoint of T3 and A1 was significantly smaller in sham controls (Dunnett's test, $*P < 0.0001$), where T3 and A1 were nearly fused. Even in the presence of injected 20E, both inhibitors halted ganglionic migration and fusion.

Fig. 8.

High-resolution images of TM-MFas II-ir somata and fascicles present within a migrating ganglion (T3), at a stage when ganglionic migration is in progress (stage P2). **A:** Confocal projection shown here (17 sections, at 3- μ m intervals) exemplifies the intense TM-MFas II labeling observed on numerous somata and fiber tracts at this stage of development. Rectangle outlines a portion of the image shown at higher magnification in B. **B:** Higher magnification image of the neuronal cluster outlined in A, showing the apparent tethering of primary neurites along thick axonal fascicles (12 optical sections at 1- μ m intervals). The punctate immunolabeling of the primary neurites and somata suggests the interpretation that TM-MFas II may be synthesized and transported through these regions, but not necessarily inserted into the membrane. Scale bars = 50 μ m in A; 100 μ m in B.

Fig. 9.

Distribution of the glycosyl phosphatidylinositol isoform of MFas II (GPI-MFas II) in the abdominal ganglia of a pupa (P2). **A:** Expression of GPI-MFas II was restricted to the surface of the ganglion (arrow) and the transverse nerve (arrowhead) in the fifth abdominal ganglion (A5). In contrast to the TM-MFas II isoform, differences in the expression of GPI-MFas II between migrating and nonmigrating ganglia were not observed ($n = 15$). Overall, GPI-MFas II expression showed little correlation with 20E titers. **Inset:** Higher magnification image of labeling on the transverse nerve. The web-like staining pattern likely corresponds to staining of glial cells within the transverse nerve, as described by Wall and Taghert (1991) and Hesterlee and Morton (2000). **B,C:** GPI-MFas II immunoreactivity shown in a single optical section (B), and a full confocal projection (C) through a region of the ganglion indicated by the arrow in B. **D:** The “ghost” of A2 is GPI-MFas II-ir. Expression is especially intense on the transverse nerve (arrowhead) and lateral nerve roots (arrow), which continue to house the axons of translocated motoneurons. **E:** Single optical section of GPI expression on the T3 ganglion of a fourth larval instar; the transverse nerve (arrowhead) and nerve roots (arrows) show GPI-MFas II-ir labeling. **F:** Single optical

section of GPI expression on the A4 ganglion of the same fourth larval instar in E; the ganglionic cortex, transverse nerve (arrowhead), and nerve roots (arrows) show GPI-MFas II-ir labeling similar to that in E. Scale bars = 100 μm in A,D; 100 μm in B (applies to B,C); 100 μm in E (applies to E,F).

Fig. 10.

Coexpression of TM-MFas II immunoreactivity and eclosion hormone (EH) immunoreactivity in the ventral nerve cord during development. **A:** Dual labeling of the ventromedial (VM) axons in T2 of a fourth instar larva; colocalization (white) of TM-MFas II (blue) and EH (red; $n = 3$). **B:** By P0, TM-MFas II immunoreactivity is present on multiple fibers per hemiganglion, shown here in T2, yet only the most medial set of fibers is doubly labeled (white, arrowhead; $n = 4$). **C:** Although TM-MFas II-ir declines during the second half of pupal–adult development, expression of TM-MFas II on the VM axons persists, shown here in the pterothoracic ganglion (fused T2–A2) of a pharate adult ($n = 8$). **D:** After adult ecdysis (eclosion), TM-MFas II immunoreactivity is not evident on the VM axons ($n = 8$). The TM-MFas II optical channel only is projected here. **E:** EH immunolabeling persists through the late adult stage ($n = 4$). The adult pterothoracic ganglion depicted here shows EH immunolabeling (single channel) 4 days after adult ecdysis. Scale bars = 150 μm .

Fig. 11.

Constitutive expression of TM-MFas II immunoreactivity by identified neuronal somata and axons. All images were collected from the same fourth larval instar. **A:** The larval brain contains four ventromedial (VM) somata (arrowhead), immunolabeled by antisera to TM-MFas II (blue) and eclosion hormone (EH; red); colabeled VM somata appear white. VM processes project laterally into the connectives linking the brain and subesophageal ganglion (SEG). The VM axons are so tightly fasciculated that each VM axonal pair appears as a single immunolabeled fiber. Additional unidentified fibers and dense neuropils also display TM-MFas II immunoreactivity but do not label with the EH antiserum (curved arrow). One bilaterally paired cluster of smaller, TM-MFas II-ir neurons are present on the dorsal surface of the brain in the protocerebrum (straight arrow). These neurons also colabeled with the TM-MFas II and EH antisera (white). **B:** Colabeled (white) descending axons of the VM neurons (arrow) are shown projecting through the SEG (connected to the brain in A). These

labeled fibers were observed extending all the way to the TAG (data not shown), consistent with the hypothesis that these neurons are the previously described VM neurons. **C:** The small colabeled somata (five cells per hemisphere) shown in A are typical of the type Ia₂ neurosecretory neurons. Their tightly fasciculated processes (arrow) are shown extending to and ramifying throughout the corpora cardiaca (CC-CA) complex. These projections are white, because they express both TM-MFas II and EH-like labeling. Scale bars = 50 μ m.