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Eph receptor expression defines midline boundaries for ephrin-positive migratory neurons in the enteric nervous system of *Manduca sexta*

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Abstract

Eph receptor tyrosine kinases and their ephrin ligands participate in the control of neuronal growth and migration in a variety of contexts, but the mechanisms by which they guide neuronal motility are still incompletely understood. Using the enteric nervous system (ENS) of the tobacco hornworm *Manduca sexta* as a model system, we have explored whether *Manduca* ephrin (MsEphrin; a GPI-linked ligand) and its Eph receptor (MsEph) may regulate the migration and outgrowth of enteric neurons. During the formation of the *Manduca* ENS, an identified set of ~300 neurons (EP cells) populate the enteric plexus of the midgut by migrating along a specific set of muscle bands that form on the gut, while they strictly avoid adjacent interband regions. By determining the mRNA and protein expression patterns for MsEphrin and the MsEph receptor and by examining their endogenous binding patterns within the ENS, we have demonstrated that the ligand and its receptor are distributed in a complementary manner: MsEphrin is exclusively expressed by the migratory EP cells, while the MsEph receptor is expressed by a discrete set of midline interband cells that are normally inhibitory to migration. Notably, MsEphrin could be detected on the filopodial processes of the EP cells that extended up to but not across the midline cells expressing the MsEph receptor. These results suggest a model whereby MsEphrin-dependent signaling regulates the response of migrating neurons to a midline inhibitory boundary, defined by the expression of MsEph receptors in the developing ENS.

Keywords

migration; guidance; enteric plexus; filopodia

INTRODUCTION

The Eph receptors are an evolutionarily conserved family of receptor tyrosine kinases that mediate cell-cell interactions in a variety of contexts during embryonic development, including tissue patterning and segmentation, neuronal outgrowth and differentiation, and angiogenesis (Wilkinson, 2001; Kullander and Klein, 2002). Eph receptors can be categorized into A or B subgroups, based on their preferential affinities for different subsets of ephrin ligands. EphA

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receptors generally bind ephrin-A ligands, which are distinguished by their glycosylphosphatidylinositol (GPI) membrane attachments. In contrast, EphB receptors generally bind ephrin-B ligands, which contain a single membrane-spanning region plus a small cytoplasmic tail (Flanagan and Vanderhaeghen, 1998; Pasquale, 2005). In the vertebrate nervous system, ephrin-Eph receptor interactions were first discovered to help form topographic maps: growing neurons expressing graded concentrations of Eph receptors are restricted by complementary gradients of ephrins in their target regions, thereby establishing an appropriate arrangement of terminal projections (Cheng et al., 1995; Drescher et al., 1997; O'Leary and McLaughlin, 2005). Alternatively, ephrin-Eph receptor interactions can define precise boundaries that confine neurons to specific regions, as has been demonstrated during rhombomere development in the hindbrain (Mellitzer et al., 1999; Cooke et al., 2001; Cooke et al., 2005) and in the guidance of neural crest cells through the somites (Krull et al., 1997; Wang and Anderson, 1997).

However, multiple ephrins and Eph receptors are often expressed in overlapping patterns within the vertebrate nervous system, and considerable promiscuity has been documented in the interactions between different ligand and receptor classes (Himanen et al., 2004; Poliakov et al., 2004; Davy and Soriano, 2005). This complexity has made *in vivo* analyses of specific ephrins and Eph receptors problematic, highlighting the need for simpler model systems with which to explore the role of particular ligand-receptor interactions during embryonic development (Pasquale, 2005). In *Drosophila*, a single ephrin (Dephrin) and one Eph receptor (DEph) are widely expressed by neurons in the developing CNS (Scully et al., 1999; Bossing and Brand, 2002), where they may help promote the segregation of axons during commissure formation (Scully et al., 1999; Bossing and Brand, 2002) and modulate axonal branching patterns (Boyle et al., 2006). Homologues of these proteins in the moth *Manduca sexta* (MsEphrin and MsEph) have also been shown to regulate the assortment of sensory axons in the developing olfactory lobe of the adult brain (Kaneko and Nighorn, 2003). These simpler systems thus offer an opportunity to examine how specific ephrin-Eph receptor combinations contribute to the regulation of neuronal guidance in a normal developmental context.

In this report, we have investigated the expression of MsEphrin and MsEph receptors in the developing enteric nervous system (ENS) of *Manduca*. During the formation of the ENS, an identified population of ~300 neurons (named the EP cells) migrates out along a preformed set of visceral muscle bands to form the enteric plexus of the midgut. At the same time, they strictly avoid adjacent interband regions, including the midline interband regions at the dorsal and ventral midline of the gut (Fig. 1; Copenhaver and Taghert, 1989a; Copenhaver and Taghert, 1989b; Copenhaver et al., 1996). In contrast to the insect CNS, where ephrins and Eph receptors are often expressed by the same neurons (Bossing and Brand, 2002; Kaneko and Nighorn, 2003), we found that MsEphrin and the MsEph receptor are expressed in discrete cellular compartments in the developing ENS: while the migratory neurons express MsEphrin, its receptor is confined to the midline interband cells of the midgut, delineating an inhibitory boundary across which the neurons normally never travel. These observations suggest that the ENS of *Manduca* may provide a unique preparation for exploring the mechanisms by which ephrin-Eph receptor interactions regulate neuronal migration *in vivo*.

MATERIALS AND METHODS

Animal preparation and histological analysis

Synchronized groups of *Manduca sexta* embryos were collected from an in-house breeding colony and maintained at 25°C. At this temperature, 1 hr corresponds to 1% of development (hatching = 100% of development). Embryos were staged using a combination of external and internal developmental markers and isolated in defined saline (in mM: 140 NaCl; 5 KCl; 28 glucose; 40 CaCl₂; 5 HEPES, pH 7.4; plus 0.2% 20-hydroxyecdysone, 0.1% insulin, 0.01%

penicillin-streptomycin, and 1% bovine serum albumin (BSA); after Horgan and Copenhaver, 1998). To expose the developing ENS, embryos were restrained in Sylgard-coated dissection chambers and incised dorsally before fixation (Copenhaver and Taghert, 1989b). For most histological experiments, the dissected embryos were then fixed for 1 hr in 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4) and processed as whole-mount preparations (described below). For paraffin sectioning, dissected embryos were fixed with Bouin's fixative (71% picric acid, 24% formalin, 5% glacial acetic acid; after Humason, 1979), immunostained with antibodies against *Manduca* fasciclin II (MsFas II; Wright et al., 1999), and embedded in paraffin. Microtome sections (8 μ m) were then collected on polylysine-coated glass microscope slides, cleared in SafeClear (Fisher Scientific, Pittsburgh, PA), and photographed at 100x. For transmission electron microscopy, embryos were fixed for 1 hr in 2% paraformaldehyde plus 2% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.0), treated with 1% OsO₄, dehydrated in ethanol, and then embedded in epoxy resin. Ultrathin (90 nm) sections were taken at designated locations along the midgut and isolated on Formvar-coated grids (Electron Microscopy Sciences, Fort Washington, PA). The sections were then imaged on a Jeol JEM-100CX II transmission electron microscope at magnifications ranging from 6,000–20,000X (Yang et al., 2005). Scanning electron microscopy was performed as previously described (Copenhaver & Taghert, 1989a; Copenhaver and Taghert, 1989b). Photomicrographs were then assembled into montages using Photoshop (Adobe Systems; San Jose, CA) and adjusted for brightness, contrast, and evenness of illumination.

Detection of MsEphrin and MsEph receptor mRNA

Northern blots were prepared using previously described methods (Wright et al., 1999) with the following modifications. Total RNA (for detecting MsEph receptor expression) or poly (A)⁺ mRNA (for detecting MsEphrin expression) was isolated from embryos at 65% of development, separated in denaturing conditions on formaldehyde agarose gels, and transferred to Duralon nylon membranes (Stratagene, Cedar Creek, TX). [α -³²P]CTP-labeled antisense probes were generated from a 500-bp fragment of the coding region from a cDNA clone containing the MsEph receptor sequence, or from the entire open reading frame of a cDNA clone encoding MsEphrin. Labeled probes were then hybridized for 24 hr to the membranes at 65°C for the MsEph receptor and 80°C for MsEphrin. After a series of high-stringency washes, the membranes were exposed to film for 24–72 hr.

For whole-mount *in situ* hybridization histochemistry, digoxigenin-labeled antisense and sense riboprobes were generated from templates containing the predicted open reading frames of the MsEphrin and MsEph receptor cDNA clones (inserted into pGEM-T; Promega, Madison, WI). A probe made against a cDNA clone encoding the extracellular domain of MsFas II was used as a positive control to label the migratory EP cells (Wright et al., 2000). Dissected embryos were fixed for 1 hr in PBS (pH 8.0) plus 4% paraformaldehyde (electron microscopy grade; Electron Microscopy Sciences), rinsed, and incubated with the riboprobes (1:100 – 1:250 in hybridization buffer) overnight at 60°C. After extensive rinsing, bound probes were detected using an alkaline phosphatase (AP)-conjugated anti-digoxigenin antibody (1:2000, Roche) and NBT/BCIP substrates (Bio-Rad; Hercules, CA; Horgan et al., 1995).

Detection of MsEphrin and MsEph receptor protein

An anti-peptide antibody (Aves Labs, Tigard, OR) was generated against a synthetic peptide unique to MsEphrin (KPVTKKTHKYDKTPNE) that had been conjugated to keyhole limpet haemocyanin (KLH); this peptide corresponds to aa 211–226 of MsEphrin (in its extracellular domain). For immunohistochemical detection of MsEphrin, staged, dissected but unfixed embryos were incubated with an IgY preparation of the anti-MsEphrin antiserum (1:100–1:250) in PBS (pH 7.4) plus 10% normal goat serum for 90 min at room temperature. Embryos were then rinsed in PBS, post-fixed in 4% paraformaldehyde for 1 hr, and incubated with an

Alexa-Fluor 488-conjugated anti-chicken IgY secondary antibody (1:1000; Molecular Probes, Eugene, OR). The preparations were also counterstained with an anti-MsFas II monoclonal antibody (C3; 1:20,000), which was visualized with a Cy3-conjugated anti-mouse IgG secondary antibody (1:200; Jackson Immunoresearch, West Grove, PA). The C3 monoclonal antibody was generated against an affinity-purified fraction of MsFas II (generously provided by Dr. James Nardi; see Nardi, 1992; Wright et al., 1999), and recognizes an undefined epitope within the shared extracellular domain of all MsFas II isoforms. For triple immunolabeling experiments, preparations were also stained with an anti-peptide antiserum that recognizes only the GPI-linked isoform of MsFas II (1:1000; see Wright and Copenhaver, 2000), which provides a glial-specific marker in the mature ENS. Z-stack confocal images of each fluorochrome were acquired on a Bio-Rad 1024 ES laser scanning confocal microscope, flattened and pseudo-colored, and then merged using Photoshop. Images were adjusted for brightness and contrast, as needed.

To detect MsEph receptors, a guinea pig anti-peptide antiserum (PRF&L, Canadensis, PA) was generated against the synthetic peptide APKYYRAKKDPKNIPC, corresponding to amino acids 309–324 within the extracellular domain of the protein. BSA- and KLH-conjugates of the peptide were used for sequential injections to enhance the antigen-specific response. Dissected embryos were fixed as described above and incubated with the antiserum for 24–48 hr (1:2000 in PBS (pH 7.4) plus 10% normal goat serum and 0.1% NaN₃). Optimal immunohistochemical staining was obtained with an AP-conjugated anti-guinea pig secondary (1:5000; Jackson Immunoresearch; West Grove, PA) and NBT/BCIP substrates.

To demonstrate the specificity of the anti-MsEph receptor and anti-MsEphrin antibodies, 5 ng of either MsEph-Fc or MsEphrin-Fc (described below) were separated by electrophoresis on 4–12% polyacrylamide gels (Criterion; Bio-Rad) and transferred to nitrocellulose membranes. The membranes were then incubated with the appropriate primary antibody overnight at 4°C and then with HRP-conjugated secondary antibodies, which were detected using the West Pico chemiluminescent kit from Pierce (Rockford, IL). For peptide preadsorption experiments, an aliquot of each antibody was incubated for 24 hr with its specific peptide antigen (at a 10:1 molar ratio of peptide:antibody), then centrifuged at 14,000 rpm in a microfuge for 10 min before being applied to replicate immunoblots. Aliquots of both the anti-MsEphrin antibody and the anti-MsEph receptor antibody were also pre-adsorbed with either their specific peptide epitopes or with the epitope used to generate the other antibody (as an unmatched control for non-specific pre-adsorption effects). These aliquots were then used to immunostain whole-mount preparations of staged embryos as an additional means of demonstrating the specificity of the immunoreactive patterns described in our results.

Production and purification of Fc-conjugated affinity probes

Fc tags are derived from the conserved region in the heavy chain of immunoglobulins and are useful for both purification and immunodetection of proteins prepared *in vitro* (Kaneko and Nighorn, 2003). Collection and purification of Fc conjugates containing the extracellular domains of MsEphrin and the MsEph receptor was performed as previously described (Kaneko and Nighorn, 2003), but with the following modifications. HEK293-EBNA cells (Invitrogen) were stably transformed with DNA constructs encoding the MsEphrin-Fc and MsEph-Fc fusion proteins (Kaneko and Nighorn, 2003), and maintained in DMEM (pH 7.0) supplemented with 10% fetal bovine serum (Hyclone; Logan, UT), 300 µg/ml hygromycin-B (Invitrogen; Carlsbad, CA), and 250 µg/ml G418 (Invitrogen). Stable expression of MsEphrin-Fc and MsEph-Fc was routinely monitored by immunoblot analysis using antibodies directed against human Fc (Jackson Immunoresearch). The medium of the transformed cell lines was then replaced with Opti-Mem (pH 7.0; Invitrogen) plus hygromycin-B and G418 for 7 days, after which the conditioned medium was collected and stored at –20° C. Fc fusion proteins were

subsequently isolated with 1–2 ml protein-A Sepharose affinity columns (Amersham, Piscataway, NJ). After several rinses with Opti-Mem, bound fusion proteins were eluted with 100 mM glycine (pH 3.0) into sufficient 1M Tris buffer (pH 9.0) to yield a final solution of pH 7.0. Pooled fractions were then dialyzed against sterile defined saline (pH 7.4) and stored at -20°C .

Whole mount binding assays with Fc-conjugated probes

To localize the endogenous distributions of bioavailable MsEphrin and MsEph receptors in the developing ENS, dissected but unfixed embryos were incubated overnight with a 20 $\mu\text{g}/\text{ml}$ solution of either MsEph-Fc, MsEphrin-Fc, or human Fc (Jackson ImmunoResearch) in defined saline (pH 7.4). After extensive rinsing with defined saline, the preparations were fixed in 4% paraformaldehyde for 1 hr. Bound fusion proteins were detected using HRP-conjugated anti-Fc antibodies (1:1000; Jackson ImmunoResearch), which were reacted with Tyramide Signal Amplification substrates for 4 min (Cy3-specific; Perkin Elmer; Boston, MA). Counterstaining with anti-MsFas II antibodies was performed as described above, but with Alexa-Fluor 488-conjugated anti-mouse IgG (1:1000; Molecular Probes) as a secondary antibody. The preparations were then imaged by confocal microscopy.

Insect Genome BLAST analysis

The coding domains of ephrin homologues were extracted from different insect genomes using standard BLAST techniques through the National Center for Biotechnology (NCBI) website (www.ncbi.nlm.nih.gov/). The extent of evolutionary divergence among ephrins from the different species was estimated using the Jotun Hein alignment in DNASTAR (Madison, WI).

RESULTS

EP cell migration on the midgut is excluded from the midline interband region

In previous reports, we showed that the formation of the ENS requires the migration of enteric neurons along a specific set of muscle band pathways that form on the surface of the gut (Copenhaver and Taghert, 1989a; Copenhaver and Taghert, 1990). Briefly, a population of approximately 300 neurons (the EP cells) invaginates as a group from the dorsal lip of the foregut to form a packet of post-mitotic but undifferentiated neurons at the foregut-midgut boundary, a process that is complete by 40% of development. During the next 15% of development (between 40–55%), the EP cells spread bilaterally around the foregut, whereupon subsets of the neurons align with one of eight longitudinal muscle bands (“b”; Fig. 1A, D) that coalesce on the adjacent midgut surface. Concurrently, interdigitation of the underlying midgut epithelial cells and muscle cells at the dorsal midline completes the closure of the gut (cf. Stark et al., 1997). Between 55–60% of development, most of the EP cells then rapidly migrate posteriorly along the eight muscle bands on the midgut (Fig. 1B, E), while a smaller number of neurons migrate onto circular muscles on the lateral foregut (circular muscles not shown in Fig. 1A–C).

Although each migratory EP cell extends an array of exploratory filopodia in advance of its leading process (Horgan and Copenhaver, 1998; Swanson et al., 2005), the neurons remain confined to their muscle band pathways while avoiding adjacent interband regions. In particular, the neurons never cross the midline interband regions of the midgut (“ml”; Fig. 1A, D, G), which at this stage is occupied by protrusions of cells within the underlying layer of circular muscle (Fig. 1. E, F). As illustrated in a transverse section of the midgut (Fig. 1G), a similar relationship is established on its ventral surface, where neurons migrating along the ventral pair of muscle bands (L4 & R4) also avoid crossing the midline. By 65% of development, the EP cells have completed their migratory dispersal along their muscle band pathways (Fig. 1C, F), but they continue to extend axons along the bands for another 10–15%

of development; Copenhaver and Taghert, 1989b). Intriguingly, although these neurons will eventually extend terminal branches onto the lateral musculature (Copenhaver and Taghert, 1989a), they continue to avoid the midline interband regions throughout the remainder of embryogenesis (described below).

To explore the cellular structure of this midline region in more detail, we performed transmission electron microscopy on sections of the embryonic midgut that were isolated during the period of EP cell migration (55–65% of development; see boxed region in Fig. 1G for orientation). As shown in figure 1H, micrographs of the midline interband region revealed the presence of a morphologically distinct set of midline cells (“**ml**”), interposed between the circular muscle cells (“**c**”) that encircle the midgut underneath the longitudinal muscle bands (“**b**”). Although these midline cells histologically resemble the adjacent circular muscle cells, they are oriented longitudinally and extend a number of small, interdigitating processes up to the surface of the gut, corresponding to the protrusions seen within the midline region in scanning electron micrographs (Figs. 1D, E, F). The strict avoidance of this region by the EP cells suggests that molecular cues expressed by these midline cells establish a non-permissive environment for the migrating neurons, thereby inhibiting abnormal midline crossing events.

Migrating EP cells express a single mRNA species encoding Msephrin

We previously showed that both the EP cells and their muscle band pathways express the homophilic cell adhesion molecule MsFas II, and that interfering with MsFas II expression or function inhibits EP cell migration and outgrowth onto the midgut (Wright et al., 1999; Wright and Copenhaver, 2000). However, in these studies, we noted that the neurons and their processes still remained confined to their normal muscle band pathways, indicating the presence of inhibitory cues on the adjacent interband regions that prevent ectopic migration and outgrowth. One candidate group of guidance molecules that might restrict the EP cells from these inappropriate environments are the Eph receptors and their ephrin ligands. In *Drosophila*, only a single Eph receptor (DEph) has been identified that interacts with a single ephrin (Deprhin), a transmembrane (class-B) ephrin that has a unique N-terminal extension (Bossing and Brand, 2002). In *Manduca*, one Eph receptor (MsEph) and one ephrin (MsEphrin) were also recently identified (Kaneko and Nighorn, 2003), but unexpectedly, MsEphrin was found to be GPI-linked, analogous to the vertebrate class-A ephrins. When we searched available genomic data for fruit flies (*Drosophila melanogaster*, *D. pseudoobscura*), mosquitoes (*Anopheles gambiae*), honeybees (*Apis mellifera*), and silkworms (*Bombyx mori*) for related sequences, we discovered that each species appears to express only a single Eph receptor with similar predicted structures (data not shown). Each species also expresses only a single ephrin ligand, but intriguingly, the membrane attachments of these molecules differ in a manner that corresponds to the evolutionary divergence of the species examined. As shown in figure 2A, while Lepidopteran and Hymenopteran species each encode a GPI-linked ephrin, Dipterans express a single transmembrane isoform. Although the developmental significance of this distinction remains to be determined, these data support the conclusion that each insect species expresses a single ephrin-Eph receptor pair, simplifying an *in vivo* analysis of their function.

To examine the expression pattern of the MsEphrin gene in the developing ENS, we probed poly-A⁺ mRNA from whole embryos in Northern blots with ³²P-labeled antisense probes specific for MsEphrin. Using this technique, we routinely detected a single band of approximately 5 kb in mRNA from embryos collected throughout the period of EP cell migration (Fig. 2B). Since previous studies on developing adult moths identified an MsEphrin-specific mRNA species of approximately 12 kb (Kaneko and Nighorn, 2003), alternate splice variants of this gene may be expressed in post-embryonic stages of development. Nevertheless, our results support the conclusion that only one primary transcript encoding MsEphrin is expressed during embryogenesis.

Using the same cDNA template containing the MsEphrin sequence, we next generated digoxigenin-labeled antisense riboprobes to examine the developmental expression of MsEphrin mRNA in the ENS by whole-mount *in situ* hybridization histochemistry (Fig. 2D–F). Identically staged sets of embryos were also hybridized with MsFas II-specific riboprobes to reveal the distribution of the EP cells at each developmental stage (Fig. 2G–I; Wright et al., 1999). MsEphrin mRNA first became detectable in the EP cells at ~50–53% of development (Fig. 2D), a stage when the neurons have spread bilaterally around the foregut but have not yet commenced their migratory dispersal (see Fig. 1A). During their subsequent migration onto the midgut (55–65% of development), all of the EP cells continued to exhibit strong levels of MsEphrin mRNA (Fig. 2E), whereas there was no detectable signal in the underlying muscle bands or adjacent interband regions. This neuronal-specific pattern of expression persisted throughout their subsequent period of axonal outgrowth along the muscle bands (65–80%; Fig. 2F). No anti-digoxigenin immunoreactivity was observed in preparations treated with sense probes generated from the same MsEphrin template (Fig. 2J; a 65% embryo is shown). These results indicate that the EP cells express the MsEphrin gene during their most active phases of motility and outgrowth in the developing ENS.

MsEphrin can be detected in the motile processes of the migratory EP cells

To analyze the expression of MsEphrin isoforms in the developing ENS, we generated an anti-peptide antibody against MsEphrin (see methods). When applied to immunoblots of recombinant Fc fusion proteins (Fig. 2C), this antibody selectively recognized MsEphrin-Fc (lane 2) but not MsEph-Fc (lane 3). Pre-adsorption with its peptide epitope blocked all binding activity (Fig 2C, lanes 4–5), further demonstrating its specificity.

Using this antibody, we next examined the pattern of MsEphrin protein expression in the developing ENS by immunostaining staged embryos throughout the period of EP cell migration and outgrowth. These preparations were also routinely counterstained with anti-MsFas II antibodies to reveal the EP cells and their muscle band pathways. Previous studies showed that MsFas II can be detected on both the somata and processes of the EP cells at 54% of development, prior to their migration (Wright et al., 1999; Wright and Copenhaver, 2000). We could also readily detect MsEphrin on the surface of the EP cells at this stage (Fig. 3B). Surprisingly, while the distribution of MsEphrin overlapped considerably with that of MsFas II, our anti-MsEphrin antibody also labeled regions of the EP cells that were devoid of MsFas II (Fig. 3C), including diffuse staining across their somata and short filopodia extending laterally from the EP cell packet onto the adjacent epithelial layers of the foregut and midgut (Fig. 3B, C, arrowheads). These observations suggest that MsEphrin may be distributed more uniformly throughout the membranes of these neurons and their processes. As in our immunoblot analysis (Fig. 2C), all MsEphrin immunoreactivity was eliminated when we pre-adsorbed the anti-MsEphrin antibody with its specific peptide epitope (Fig 2L), but not when we pre-adsorbed it with an MsEph receptor-specific peptide (Fig. 2K).

During the subsequent phase of active EP cell migration (55–65% of development), MsFas II is transiently expressed by the muscle band pathways of the midgut as well as the neurons traveling along them (Wright et al., 1999; Wright and Copenhaver, 2000). In contrast, MsEphrin expression was restricted to the EP cells. Application of our anti-MsEphrin antibody to unfixed embryos (followed by rapid fixation and visualization with secondary antibodies) provided robust labeling of all of the neurons and their leading processes (Fig. 3E, H), including lamellipodial and filopodial extensions that were only faintly labeled with anti-MsFas II antibodies (Fig. 3G–I, arrowheads). While the images shown in figure 3 depict MsEphrin expression on the four dorsal migratory pathways of the midgut (L1–L2 & R1–R2), a similar pattern was observed on the four ventral pathways (see Fig. 1G), including MsEphrin-positive processes from the EP cells that extended up to but not across the ventral midline (not shown).

By 65% of embryogenesis, the EP cells have completed their migration but continue to extend axons posteriorly along the muscle bands for another 20% of development (Copenhaver and Taghert, 1989b). During this period, MsFas II becomes increasingly localized to the growing axons of the EP cells, while it is down-regulated in the underlying muscle bands (Wright and Copenhaver, 2000). In contrast, we found that MsEphrin continued to be distributed uniformly throughout the EP cells, providing robust labeling of both their somata and growing processes (Fig. 4B, E, H). In particular, anti-MsEphrin staining revealed a population of fine filopodial processes that extended from the EP cells up to but not across the midline interband region between the dorsal muscle bands (Fig. 4E–F, arrowheads). More posteriorly on the midgut, the fasciculated axons and leading growth cones of the EP cells could also be readily distinguished with both antibodies, including filopodial protrusions (relatively enriched with MsEphrin) that extended over the bands and up to the midline interband cells (Fig. 4 H and I, arrowheads).

MsEphrin is specifically expressed by neurons and not glia in the developing ENS

Once the EP cells have achieved their mature positions on the foregut and midgut and have begun to extend processes onto the adjacent visceral musculature (Copenhaver and Taghert, 1989a; Copenhaver and Taghert, 1989b), a subsequent wave of migratory glial cells ensheathes the major branches of the enteric plexus (Copenhaver, 1993). This glial population can be distinguished by their expression of the GPI-linked isoform of MsFas II, while the EP cells and their muscle band pathways express only transmembrane isoforms of MsFas II at this stage of development (Wright and Copenhaver, 2000). We therefore asked whether the enteric glia of the ENS also express MsEphrin. Double-immunostaining preparations at 80% of development revealed that neurons on the midgut band pathways continued to express both MsFas II, which was localized primarily to their axons and terminal branches (Fig. 5A; green in Fig. 5D, E, H), and MsEphrin, which remained distributed throughout their somata and processes (Fig. 5B; magenta in Fig. 5D; red in Fig. 5F, H). Intriguingly, the subsets of EP cells that had migrated laterally onto the radial musculature of the foregut also continued to stain strongly for MsEphrin but no longer expressed detectable levels of MsFas II (see asterisks in Fig. 5B, D, F, H).

As previously shown (Copenhaver, 1993), a delayed wave of glial cells spreads along the major branches of the enteric plexus to ensheath the EP cells, once neuronal migration in the ENS is complete. Unlike the EP cells (which express the transmembrane form of MsFas II), the enteric glial cells express only the GPI-linked isoform of this receptor (Wright and Copenhaver, 2000). When we also immunostained preparations with antibodies against GPI-MsFas II (Fig. 5C; blue in Fig. 5G, H), we found that the ensheathing glial cells were clearly distinguishable from the MsEphrin-positive sets of neurons on both the foregut and the midgut. These distinct patterns of expression were more readily apparent at higher magnification (Fig. 5I–L; equivalent to the boxed regions in Fig. E–H): while our antibody against the shared extracellular domain of MsFas II labeled both the EP cells and their ensheathing glia (green), as well as their underlying muscle band pathways (b), MsEphrin expression was restricted to the EP cells and their filopodial processes (red), while GPI-MsFas II was confined to the elaborating processes of the glial cells (blue). These results demonstrate that MsEphrin is expressed in a neuronal-specific manner throughout ENS development, providing a robust marker for the EP cells and their growing processes (including their exploratory filopodia) as they navigate to their mature locations on the foregut and midgut musculature.

MsEph receptors are localized to the midline interband cells of the midgut

The sole Eph receptor homologue in *Manduca* (MsEph) has all of the characteristic features of Eph receptors identified in other systems: its primary amino acid sequence aligns equally well with both the A and B Eph receptor subclasses found in vertebrates, and it has been shown

to act as an authentic receptor for MsEphrin (Kaneko and Nighorn, 2003). Using 32 P-labeled riboprobes against the coding domain of the MsEph receptor sequence, we detected a single labeled band of ~8 kb in Northern blots of total RNA collected from embryos at 65% of development (Fig. 6A). This result indicates that, like MsEphrin, only a single transcript encoding the MsEph receptor is expressed during the period of EP cell migration and outgrowth. In contrast, multiple mRNA species encoding MsEph receptors have been detected in the brain of *Manduca* during metamorphosis (Kaneko and Nighorn, 2003), again suggesting that MsEphrin-MsEph receptor interactions during post-embryonic development may involve a more complex array of ligand and receptor isoforms than seen in the embryonic nervous system. Using digoxigenin-labeled antisense riboprobes against the MsEph receptor sequence, we next examined the expression of this gene in the developing ENS by whole-mount *in situ* hybridization histochemistry. MsEph receptor-specific mRNA was localized exclusively to the midline interband cells positioned between the mid-dorsal pair of muscle bands (Fig. 6C), as well as the symmetrically oriented ventral midline cells (not shown). In contrast, MsFas II mRNA was strongly expressed in the muscle band pathways of the midgut (Fig. 6E), as well as the EP cells (Fig. 2; previously reported in Wright et al., 1999). No specific labeling of the dorsal and ventral midline cells was detected when we applied sense control probes generated from our MsEph cDNA clone (Fig. 6G).

To validate this result, we generated an anti-peptide antiserum against the extracellular domain of the MsEph receptor (see methods). As shown in figure 6B, this antiserum labeled MsEph-Fc fusion proteins (lane 3) but not MsEphrin-Fc (lanes 2). All specific activity was eliminated by pre-adsorption of the antiserum with its MsEph receptor-specific peptide epitope (lanes 4–5), further demonstrating its specificity. As seen in our *in situ* hybridization analysis (Fig. 6C), immunostaining the ENS with our anti-MsEph receptor antiserum labeled only the midline interband cells between the dorsal muscle bands (Fig. 6D) and between the ventral muscle bands (not shown). In contrast, MsFas II immunoreactivity was confined to the adjacent muscle bands (Fig. 6F) and EP cells (Figs. 3–4). Pre-adsorption of the anti-MsEph antibody with its peptide epitope eliminated all immunostaining in the midline regions (Fig. 6H), while pre-adsorption with the MsEphrin-specific peptide had no effect (not shown). Thus, the expression of MsEph receptor mRNA and protein by the midline interband cells complements the expression of MsEphrin by the migratory neurons. Due to the different staining conditions required for our anti-MsEphrin and anti-MsEph receptor antibodies, we were unable to use them simultaneously to double-immunostain the same preparations. Nevertheless, our results show that the patterns of expression of the ligand and its receptor were clearly distinct, suggesting that MsEphrin-MsEph receptor interactions may play a role in regulating the guidance of the EP cells at the midline during their migration and outgrowth.

Fc fusion proteins of MsEphrin and MsEph recognize endogenous binding partners *in vivo*

To demonstrate that the patterns of MsEphrin and MsEph receptor expression described in the foregoing sections represent functional ligand and receptor distributions in the developing ENS, we generated MsEphrin-Fc and MsEph-Fc fusion proteins (after Kaneko and Nighorn, 2003). First, to test whether MsEphrin-Fc could bind endogenous MsEph receptors *in vivo*, we incubated embryos that had been dissected to expose the ENS with serum-free medium containing 20 µg/ml MsEphrin-Fc overnight. The preparations were then fixed and immunostained with anti-Fc antibodies (magenta; to reveal where the fusion protein had bound) and with anti-MsFas II antibodies (green; to delineate the EP cells and their muscle band pathways). As shown in figure 7A–F, MsEphrin-Fc bound specifically to the midline interband regions along the entire length of the midgut between the dorsal muscle bands, both in the vicinity of the migrating EP cells (Fig. 7A–C) and more posteriorly between their growing axons (Fig. 7D–F). A similar pattern of labeling was seen at the ventral midline of the gut (not

shown). This distribution of bound MsEphrin-Fc precisely matched the expression pattern of MsEph receptor-specific mRNA and protein, as shown in figure 6C–D.

Conversely, when we incubated embryos with 20 $\mu\text{g/ml}$ MsEph-Fc to detect endogenous MsEphrin (Fig. 7G–L), both the EP cells (Fig. 7G–I) and their leading processes (Fig. 7J–L) were selectively labeled, but not the underlying muscle bands or the midline interband cells. Again, the distribution of bound MsEph-Fc directly corresponds to the pattern of MsEphrin expression in these neurons, as revealed by *in situ* hybridization and immunohistochemical methods (Figs. 2–4). Control Fc proteins applied to cultured embryos at a similar concentration resulted in no specific staining (Fig. 7M–R), further demonstrating the specificity of our constructs for their endogenous binding partners. Thus, the *in vivo* binding patterns of these fusion proteins directly correspond to the endogenous distributions of the MsEphrin ligand and its MsEph receptor.

DISCUSSION

In this study, we examined the developmental expression of MsEphrin and its receptor (MsEph) in the developing ENS of *Manduca*, and found that the two proteins are localized in a complementary pattern: whereas the migratory neurons that populate the midgut enteric plexus express MsEphrin, a discrete set of midline cells adjacent to their muscle band pathways express the MsEph receptor. Besides our characterization of MsEphrin and MsEph receptor expression by *in situ* hybridization and immunohistochemical methods, we also verified the endogenous binding sites for the ligand and its receptor using Fc-fusion constructs. As predicted from their patterns of expression, MsEphrin-Fc proteins bound specifically to the midline interband cells (corresponding to endogenous distributions of the MsEph receptor), while MsEph-Fc proteins bound the EP cells (corresponding to their expression of MsEphrin). These *in vivo* binding experiments further validate our analysis of the expression patterns for these proteins, in that they demonstrate that both the ligand (on the EP cells) and the receptor (on the midline interband cells) represent bioavailable pools of MsEphrin and MsEph receptors that can potentially regulate the guidance of the neurons and their processes.

A possible role for MsEphrin-MsEph receptor interactions in the developing ENS

The discrete localization of the MsEph receptor to the midgut midline cells and MsEphrin to the migratory EP cells presents a striking contrast to the distribution of other guidance cues in the developing ENS, suggesting a specific role for MsEphrin-MsEph receptor interactions in controlling EP cell guidance. Previous studies have shown that neuronal migration in the developing ENS is precisely regulated: the EP cells and their processes can travel onto any of the eight midgut muscle bands (which appear to form equivalent pathways) but are strongly repelled from the adjacent interband regions, indicating the presence of regionally localized guidance cues that mediate these directional responses (Copenhaver et al., 1996). One of the cues that promotes EP cell migration along the band pathways is MsFas II, a homophilic adhesion receptor related to vertebrate NCAM and OCAM that is expressed by both the neurons and their band pathways during the migratory period (Figs. 2, 7; Wright et al., 1999; Wright and Copenhaver, 2000). Manipulations that interfered with MsFas II-dependent adhesion inhibited the migration and outgrowth of the EP cells along the bands, but they did not result in ectopic migration onto the adjacent interband regions (Wright et al., 1999; Wright and Copenhaver, 2000). Hence, other inhibitory guidance cues must restrict the EP cells from these inappropriate domains.

Our current studies indicate that at the midline, the MsEph receptor is a likely candidate for mediating this process. Live-embryo staining with our anti-MsEphrin antibody revealed that this ligand is abundantly expressed by the migratory EP cells and their leading processes (Figs. 3, 4), including exploratory filopodia that extend up to but not across the midline interband

cells, where the MsEph receptor is localized (Figs. 6, 7). Previous studies showed that these midline cells can be distinguished by a variety of antigenic markers (Horgan et al., 1995; and unpublished data), but their identity and function remained unclear. The histological and electron microscopic analysis presented in this paper (Fig. 1) indicates that this midline region of the embryonic midgut is formed by a morphologically distinct set of cells interposed between the circular muscles of the midgut. Although these midline cells resemble the circular muscle cells in diameter and cytological appearance in electron micrographs, they are oriented longitudinally along the dorsal and ventral midline of the gut, and they do not appear to become overgrown by the longitudinal muscle fibers that form elsewhere around the gut surface (including the cells that condense into the eight muscle bands). These midline cells also elaborate a dense meshwork of short, interdigitating processes during midgut closure, which is retained in the form of a compact lattice of membranous extensions once gut closure is complete. The outer surface of these midline cells therefore form the most superficial layer of the midgut at the dorsal and ventral midline. Any membrane-associated receptors that are expressed by these cells (such as the MsEph receptor) would therefore be readily accessible to the exploratory filopodia of the migratory EP cells.

Given the abundant precedent of ephrin-Eph receptor interactions in mediating repulsive responses by growing neurons (Wilkinson, 2001; Kullander and Klein, 2002), the pattern of MsEphrin and MsEph receptor expression in the developing ENS suggests the following model (Fig. 8). As the migratory EP cells travel along their muscle band pathways (green), they extend exploratory filopodia expressing MsEphrin (blue) onto the bands and the adjacent interband regions. When these filopodia extend onto the midline interband regions (pink), they encounter MsEph receptors (red) on the surface of these cells. MsEphrin-MsEph receptor interactions then promote a local retraction response by these filopodia, thereby helping to steer the migrating neurons away from the midline region.

Unlike other instances where motile neurons expressing Eph receptors are repelled by ephrin ligands (Krull et al., 1997; Wang and Anderson, 1997; Knoll et al., 2001), we found that the EP cells express MsEphrin, while the midline interband cells express the MsEph receptor. These results suggest that in the developing ENS, guidance of the migratory EP cells is mediated via *reverse* signaling by MsEph receptors to induce MsEphrin-dependent changes in filopodial behavior, as opposed to *forward* signaling by an ephrin ligand via their cognate Eph receptors. Reverse signaling via B-type (transmembrane) ephrins has been well established in a variety of systems (Kullander and Klein, 2002; Davy et al., 2004). Reverse signaling through GPI-linked ephrins (like MsEphrin) has also been implicated in several instances (Knoll and Drescher, 2002; Holmberg et al., 2005), although the mechanism underlying this process remain controversial (Davy and Soriano, 2005). Alternatively, forward signaling by neuronal MsEphrin via MsEph receptors on the midline cells might result in the release of a secondary factor that in turn inhibits growth and migration across the midline. The experimental accessibility of the developing ENS of *Manduca* can now be used to test whether MsEph-dependent interactions do indeed control neuronal migration at the midline, and to explore the signal transduction pathways that may be modulated by MsEphrin to regulate neuronal motility *in vivo*.

As shown in figures 3–4, we found that MsEphrin was robustly expressed by the EP cells that migrated onto the dorsal and ventral band pathways (bands L1 & R1 and L4 & R4; see Fig. 1), adjacent to the midline interband regions where the MsEph receptor is expressed. However, MsEphrin was also expressed by EP cells traveling onto the more lateral muscle bands of the midgut (L2 & L3 and R2 & R3; Fig. 3) and onto the radial muscle pathways of the foregut (Fig. 4, asterisks), regions that are not associated with cells expressing the MsEph receptor. In *Caenorhabditis elegans*, GPI-linked ephrins may have biological roles that are independent of Eph receptor interactions (Chin-Sang et al., 1999; Gauthier and Robbins, 2003), and MsEphrin

might likewise regulate interactions with other binding partners besides the MsEph receptor. Alternatively, since the specific pathway chosen by an individual EP cell varies from animal to animal (Copenhaver and Taghert, 1989a; Copenhaver and Taghert, 1989b; Copenhaver et al., 1996), the global expression of MsEphrin by all of the EP cells may simply ensure that any neuron encountering MsEph receptors at the midline will respond appropriately.

At the gross morphological level, the ENS of invertebrates clearly differs from that of vertebrates, but they share a number of key structural and functional features. In both phyla, the ENS consists of small, interconnected ganglia and branching nerve plexuses that innervate the smooth muscle of the gut, providing autonomic control to the digestive tract. Analogies may be drawn between their developmental origins, as well. In both systems, enteric neurons and glia are generated from neurogenic epithelia and must subsequently migrate considerable distances to reach the developing gut. In vertebrates, the cells of the ENS are derived from the vagal and sacral neural crest (Le Douarin and Kalcheim, 1999; Burns, 2005), while in insects, neural precursors of the ENS invaginate from neurogenic placodes in the ectodermal foregut (Copenhaver and Taghert, 1990; Copenhaver and Taghert, 1991; Hartenstein et al., 1994; Ganfornina et al., 1996). In this regard, the developmental origins of the insect ENS also resemble the generation of cranial sensory ganglia of vertebrates from neurogenic placodes (Streit, 2004).

Our work in *Manduca* has shown that many of the same classes of guidance cues involved in controlling neural crest cell migration also regulate the pathways chosen by the EP cells, including immunoglobulin-related cell adhesion receptors (Wright and Copenhaver, 2000; Yoneda et al., 2001; Anderson et al., 2006) and integrins (Breau et al., 2006; Coate & Copenhaver, unpublished data). While Ephrin-Eph receptor interactions play a prominent role in controlling the migration of trunk neural crest cells (Krull 2001), whether they also help guide crest-derived enteric neurons has not been comprehensively explored. To our knowledge, this report represents the first description of ephrin-Eph receptor expression in the developing ENS of any system.

The ENS of *Manduca* as a model system for ephrin-dependent control of migration

The regulation of neuronal growth by complementary patterns of ephrin ligands and Eph receptors is a recurring theme in embryonic development (e.g. Krull et al., 1997; Wang and Anderson, 1997; Davy and Soriano, 2005). In several regions of the vertebrate brain, neurons expressing graded patterns of Eph receptors are guided by complementary gradients of ephrins, resulting in the formation of topographically arrayed axonal projections (Cheng et al., 1995; Drescher et al., 1995; O'Leary and Wilkinson, 1999; O'Leary and McLaughlin, 2005). Alternatively, ephrins and Eph receptors can also regulate the establishment of discrete boundaries that restrict the movement of cells or growing processes (reviewed by Wilkinson, 2001). For example, the migration of neural crest cells expressing EphB receptors is restricted to the rostral half of each sclerotome segment by the expression of type B ephrins in the caudal half (Krull et al., 1997; Wang and Anderson, 1997), although at later stages, type B ephrins actually attract neural crest cells into the adjacent dorsomedial mesenchyme (Santiago and Erickson, 2002).

However, determining the role of specific ephrin-Eph receptor interactions in vertebrates has been hindered by the presence of multiple ligand-receptor combinations that are expressed in overlapping patterns and exhibit considerable promiscuity in their interactions (Himanen et al., 2004; Poliakov et al., 2004; Davy and Soriano, 2005). As already noted, insect systems express only a single ephrin-Eph receptor combination, greatly simplifying *in vivo* analyses of their developmental functions. Intriguingly, in the insect CNS, ephrins and their receptors are often co-expressed, suggesting a possible role for reciprocal interactions among developing neurons. In *Drosophila* Dephrin and its receptor (DEph) are both expressed within the neurons

that form the commissural tracts of the ventral nerve cord and the mushroom bodies of the brain, although the ligand and receptor appear to have complimentary subcellular distributions (Bossing and Brand, 2002; Boyle et al., 2006). Similarly in *Manduca*, both MsEphrin and the MsEph receptor are expressed by axons growing into the olfactory glomeruli of the brain, although again the proteins exhibit complementary distributions (Kaneko and Nighorn, 2003). In contrast, our results suggest that in the developing ENS, the sharply defined expression of the MsEph receptor by the dorsal and ventral midline cells of the midgut may serve to prevent inappropriate growth of the MsEphrin-expressing EP cells across these boundaries. This preparation may therefore provide a unique opportunity to investigate the potential role of signaling via a GPI-linked ephrin in the control of neuronal guidance within a developing nervous system.

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Figure 1.

The EP cells migrate along pre-formed muscle bands but avoid the midline interband region of the midgut. (A–C) Schematic drawings from scanning electron micrographs illustrate the progression of EP cell migration along the dorsal four muscle bands of the midgut. (A) By 55% of embryonic development, the EP cells have emerged from a neurogenic placode on the foregut and have spread bilaterally to encircle the foregut-midgut boundary (FG/MG). Dorsal closure of the midgut involves the extension of interdigitating processes from adjacent epithelial and muscle cells to occupy the midline interband region (ml; hatched area). Concurrently, as the midgut closes, subsets of longitudinal muscle cells on the midgut surface coalesce into eight identifiable muscle bands (b); only the dorsal four bands are shown (L1–L2 & R1–R2). Groups of EP cells (black) align with each of these bands, which will subsequently serve as migratory pathways for the neurons. (B) By 58% of development, the EP cells have begun to migrate posteriorly along the midgut bands (arrows), extending

processes that explore the midgut surface but do not cross the midline region. Some neurons also migrate laterally out along radial muscle fibers on the foregut (foregut muscles not shown). (C) By 65% of development, the EP cells have completed their migration but continue to extend axons posteriorly along the muscle bands while avoiding the midline interband region. (D–F) Scanning electron micrographs highlighting the dorsal pair of muscle band pathways (L1 & R1) that are followed by the EP cells on the midgut. (D) Magnified view of the boxed region in panel A shows the muscle cells that are coalescing into the dorsal pair of band pathways (**b**), and the interdigitating processes of cells that occupy the midline interband region (**ml**). (E) The corresponding region of panel B, showing the groups of EP cells that have begun to migrate on the bands (arrows). (F) The corresponding region in panel C, showing the post-migratory EP cells that have become distributed along the bands. Note that the midline interband region now consists of a narrow stripe of interdigitating processes that are still avoided by the EP cells. (G) Paraffin section of an isolated midgut from a 65% embryo, immunostained for MsFas II (section was taken at approximately the position of the line (*) in panel C). All eight longitudinal band pathways (L1–L4 and R1–R4) can be distinguished on the surface of the midgut (D = dorsal; V = ventral; **ml** = the mid-dorsal and mid-ventral interband regions). (H) Transmission electron micrograph of a transverse section of the midgut, showing the dorsal pair of bands (L1 & R1) and the midline interband cells (equivalent to the boxed region in G). Subsets of neurons (**n**) can be distinguished that have migrated onto the underlying muscle band cells (**b**). The midline interband cells (**ml**) form a morphologically distinct set of longitudinally oriented cells (cut in cross-section) that are interposed between adjacent circular muscle cells (**c**), which in turn underlie the more superficial longitudinal muscles of the midgut. Scale bars for A–C, 40 μm ; D–F, 30 μm ; H, 10 μm .

Figure 2.

A single isoform of MsEphrin is expressed by the migratory EP cells. (A) Dendrogram showing the evolutionary relationship of ephrins from different insect species, based on predicted amino acid similarities. The genome of each species contains only a single ephrin gene, although the predicted proteins differ in their membrane attachments (GPI = glycosyl phosphatidylinositol-linked; TM = transmembrane). Scale = amino acid substitutions X 100. NCBI Accession numbers of the sequences used for this analysis are follows: for *Manduca sexta* (MsEphrin): AAQ67232; for *Bombyx mori* : BAAB01017201; for *Apis mellifera* , XP 392239; *Anopheles gambiae*, EAL42045; for *Drosophila melanogaster* (Dephrin): NP 726585. All sequences were conceptual translations from cDNA clones except for *Bombyx mori* ephrin, which was computationally predicted from genomic DNA. (B) Northern Blot of embryonic mRNA with a riboprobe specific for MsEphrin mRNA reveals a single band at approximately 5 kb (arrow). (C) Immunoblot of Fc fusion proteins containing the extracellular domains of

MsEphrin and the MsEph receptor demonstrates the specificity of our anti-MsEphrin antibody. Lanes 1, 2, & 4 were each loaded with 5 ng of MsEphrin-Fc; lanes 3 and 5 were loaded with 5 ng of MsEph-Fc. Lane 1: an antibody against human Fc labels MsEphrin-Fc. Lanes 2–3: the anti-MsEphrin antibody recognizes MsEphrin-Fc (lane 2) but not MsEph-Fc (lane 3). Lanes 4–5: an aliquot of the anti-MsEphrin antibody that was pre-adsorbed with its peptide epitope produced no labeling of either MsEphrin-Fc (lane 4) or MsEph-Fc (lane 5). (D–F) Staged embryos stained by whole mount *in situ* hybridization histochemistry with a riboprobe specific for MsEphrin; D = 55%, E = 58%, and F = 65% of development. All of the EP cells but not the muscle bands are labeled throughout this developmental period. Scale bar = 30 μ m. (G–I) An age-matched set of embryos labeled with a riboprobe specific for MsFas II shows the positions of the EP cells throughout the migratory period. (J) An embryo at 65% of development treated with an MsEphrin-specific sense control riboprobe shows no anti-digoxigenin immunoreactivity. (K) Pre-adsorption of the anti-MsEphrin antiserum with an MsEph receptor-specific peptide still labeled the EP cells in a 65% embryo (compare to figures 3–4). (L) In contrast, pre-adsorption of the anti-MsEphrin antiserum with its peptide epitope (specific for MsEphrin) eliminates all immunostaining. Arrows indicate the positions of the EP cells on the dorsal pair of muscle bands (L1 & R1; see Fig. 1). Scale bar = 30 μ m.

Figure 3.

MsEphrin is expressed by the EP cells and their leading processes as they migrate onto the midgut muscle bands. (A–C) Whole-mount immunostaining of the EP cells in an embryo at 54% of development (just prior to migration onset) that was double-labeled with antibodies against MsFas II (green) and MsEphrin (magenta). Arrowheads indicate short filopodial processes labeled with anti-MsEphrin but not MsFas II. (D–E) Double-immunostaining of an embryo at 60% of development shows that all of the migrating EP cells express both MsFas II and MsEphrin; fainter MsFas II staining can also be seen in the underlying midline bands (L1 & R1). (G–I) Magnified views of the boxed regions in D–F show the expression of MsFas II (green) in both the leading processes of the migrating EP cells and their underlying muscle bands (**b**); in contrast, MsEphrin staining (magenta) is absent from the muscle band cells but reveals the full extent of the EP cell processes, including filopodia (arrowheads) that extend over the muscle bands but not across the interband midline cells (**ml**). Scale bar = 30 μ m in

A–F and 10 μm in G–I; en = esophageal nerve of the foregut; FG/MG = foregut/midgut boundary.

Figure 4.

MsEphrin continues to be expressed by the EP cells and their growing axons once migration is complete. (A–C) Whole-mount immunostaining of the EP cells in an embryo at 65% of development (at the end of EP cell migration) that was double-labeled with antibodies against MsFas II (green) and MsEphrin (magenta). (D–F) Magnified views of the boxed regions in A–C show that MsFas II is increasingly localized to the growing axons of the EP cells, while MsEphrin remains uniformly distributed throughout their cell bodies and processes. MsEphrin immunostaining also clearly labels EP cell filopodia (arrowheads) that extend across the MsFas II-positive muscle bands (**b**) but not onto the midline cells (**ml**). (G–I) A more posterior segment of the midgut at 65% of development (different preparation than in A–F) shows MsFas II and MsEphrin immunostaining in the fasciculated axons and leading growth cones of the EP cells on the muscle bands (**b**). MsEphrin-positive filopodia (arrowheads) extend towards but not across the midline interband cells (**ml**). Scale bars = 30 μ m in A–C and 10 μ m in D–I. FG/MG = foregut/midgut boundary.

Figure 5.

MsEphrin is expressed in a neuronal-specific manner in the mature ENS. An embryo at 80% of development was triple-immunostained with antibodies against the extracellular domain of MsFas II (shared by all MsFas II isoforms), MsEphrin, and the GPI-linked isoform of MsFas II as a glial-specific marker (Wright & Copenhaver, 2000). Black and white images of the individual staining patterns are shown in A–C, and a merged image of MsFas II (green) and MsEphrin (magenta) is shown in D. For triple-labeling, MsFas II is shown in green, MsEphrin is shown in red, and GPI-MsFas II is shown in blue. (A, E) Immunostaining with a pan-MsFas II antibody labels both the EP cells and their underlying muscle bands (L1 & R1; which express transmembrane MsFas II) as well as the ensheathing glial cells (which express GPI-linked MsFas II). TM-MsFas II in the neurons is largely confined to their growing processes at this stage. (B, F) MsEphrin continues to be strongly expressed throughout EP cells and their processes on both the foregut and midgut. (C, G) An antibody specific for the GPI-linked

isoform of MsFas II selectively stains migratory glial cells that are ensheathing the major branches of the enteric plexus. (D) A merged image shows that while the distribution of MsFas II (green) and MsEphrin (magenta) in the EP cell processes largely overlap, MsEphrin is also strongly expressed in the neuronal somata, including subsets of EP cells that have migrated onto the radial muscles of the foregut (asterisks). (C, G) An antibody specific for the GPI-linked isoform of MsFas II selectively stains a population of migratory glial cells that are ensheathing the major branches of the enteric plexus. (H) A merged image of all three channels shows that MsEphrin staining (red) overlaps with transmembrane MsFas II in the growing axons of the EP cells but does not overlap with GPI-linked MsFas II in the glial cells (blue), indicating that MsEphrin is only expressed by the neurons of the ENS. I–L: higher magnification of the boxed regions indicated in E–H. Scale bar = 30 μm for A–H and 10 μm for I–L.

Figure 6.

A single mRNA species encoding the MsEph receptor is expressed by the midline interband cells. (A) Northern Blot of total RNA from embryos labeled with 32 P-labeled antisense riboprobes specific for MsEph receptor mRNA reveals a single band at ~8.0 kb. (B) Immunoblot of Fc fusion proteins containing the extracellular domains of MsEphrin and the MsEph receptor demonstrates the specificity of our anti-MsEph receptor antibody. Lanes 1, 3, & 5 were each loaded with 5 ng of MsEph-Fc; lanes 2, & 4 were loaded with 5 ng of MsEphrin-Fc. Lane 1: an antibody against human Fc labels MsEph-Fc. Lanes 2–3: the anti-MsEph receptor antiserum does not recognize MsEphrin-Fc (lane 2) but does positively stain MsEph-Fc (lane 3). Lanes 4–5: an aliquot of the anti-MsEph receptor antibody that was pre-adsorbed with its peptide epitope produces no labeling of either MsEphrin-Fc (lane 4) or MsEph-Fc (lane 5). (C) Whole-mount *in situ* hybridization staining of an embryonic midgut at 65% of development (at a position posterior to the migratory EP cells) with a riboprobe specific for

MsEph receptor mRNA labels the midline interband cells (**ml**) but not the midgut muscle bands (**b**). (D) Immunostaining the midgut with the anti-MsEph receptor antiserum also stains the midline interband cells but not the bands. (E) In contrast, *in situ* hybridization labeling with a riboprobe specific for MsFas II mRNA stains the midgut muscle bands (**b**) but not the midline interband cells (**ml**). (F) Immunostaining the embryonic midgut with an anti-MsFas II antiserum produces a similar pattern of band-specific staining. (G) Sense riboprobes generated against the cDNA template encoding the MsEph receptor fail to produce any specific labeling of the midline cells. (H) Immunostaining embryos with an aliquot of the anti-MsEph receptor antiserum that had been pre-adsorbed against its MsEph-specific epitope also fails to label the midline cells. Scale bar = 30 μ m.

Figure 7.

Fc-fusion proteins of MsEphrin and the MsEph receptor label the midline cells and EP cells, respectively. (A–F) A 62% embryo that was incubated with MsEphrin-Fc, fixed, and then double-immunostained with anti-Fc (magenta) and anti-MsFas II antibodies (green). Panels A–C show the anterior midgut containing the migratory EP cells (arrows); panels D–F show their growing axons (arrowheads). MsEphrin-Fc proteins selectively bind the midline interband cells (*) that express the MsEph receptor (see Fig. 6), but not the EP cells or their band pathways (b). (G–L) A 62% embryo that was incubated with MsEph-Fc, fixed, and then double-immunostained with anti-Fc (magenta) and anti-MsFas II antibodies (green). Panels G–I show the migratory EP cells (arrows); panels J–L show their growing axons (arrowheads). MsEph-Fc proteins selectively bind the EP cells and their processes that express MsEphrin (see Figs. 2–4), but not the underlying muscle bands (b) or the midline interband cells (*). (M–R) A 62% embryo that was incubated with Fc protein as a control, fixed, and then double-immunostained

with anti-Fc (magenta) and anti-MsFas II antibodies (green). No specific Fc labeling was seen in either the EP cells (panels M–O, arrows) or their growing axons (panels P–R; arrowheads), nor in the muscle bands (**b**) and the midline interband cells (*). Scale bar = 25 μ m.

Figure 8.

A model for the regulation of EP cell migration at the ENS midline by MsEphrin-MsEph receptor interactions. Left panel: migrating EP cells extend filopodia enriched in MsEphrin (blue) along their muscle band pathways (**b**; green), as well as onto the adjacent midline cells (**ml**; pink) and the lateral interband regions (yellow). Filopodia that extend onto the midline interband cells encounter cells expressing the MsEph receptor (red). Right panel: enlarged view of the boxed region in the left panel. In step 1, an MsEphrin-positive filopodium extends onto the midline interband cells expressing the MsEph receptor. In step 2, interactions between neuronal MsEphrin and MsEph receptors at the midline induces a signaling response in the filopodium, possibly via reverse signaling (R; white arrow) through MsEphrin itself. Alternatively, MsEphrin-MsEph receptor interactions might induce a forward signaling response (F, black arrow) via the MsEph receptor to stimulate a secondary signal from the midline cells that then feeds back on the filopodium. In step 3, this interaction leads to the retraction of the filopodium off the midline interband cells. Consequently, the EP cells and their growing processes are guided away from the midline, helping to maintain their correct trajectories along the band pathways.