THE ROLE OF EPHRIN-EPH RECEPTOR SIGNALING DURING ENTERIC NERVOUS SYSTEM FORMATION IN *MANDUCA* SEXTA

by

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A DISSERTATION

Presented to the department of Cell and Developmental Biology, the Program in Molecular and Cellular Biosciences, and the Oregon Health & Sciences School of Medicine in partial fulfillment of

Doctor of Philosophy

15 April 2008

School of Medicine
Oregon Health & Science University

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ACKNOWLEDGEMENTS

I first would like to thank my advisor Dr. Philip Copenhaver for his extraordinary effort and guidance throughout this thesis. Any success I may have as a scientist will be a reflection of my being under his tutelage. I thank the members of the Copenhaver lab for their great assistance and camaraderie. In particular, I thank Ms. Tracy Swanson for her help in developing my technical skills early in this work, and for her exceptional talents in the laboratory. I also thank the members of my thesis advisory committee for their outstanding advice: Drs. David Morton (chair), Philip Copenhaver, Mike Forte, Ronen Schweitzer, and Larry Sherman. I also thank the administrative staff in the department of Cell and Developmental Biology for their constant and enthusiastic support: Dr. Bruce Magun (departmental chair), Niki Larson, Elaine Offield, and Pam Wagoner. I thank the examination committee for their critical evaluation of this thesis work: Drs. David Morton (chair), Philip Copenhaver, Doris Kretzschmar, Mike Forte, and Ronen Schweitzer. I would also like to thank my undergraduate advisor Dr. Alyce DeMarais for inspiring my interest in developmental biology. Many thanks go to my family and friends for their limitless support throughout this work. Most importantly, I thank my wife, Megan, and son, Soren. Without their love, patience, and support, this thesis would not have been a success.

ABSTRACT

In this thesis, I have examined the molecular mechanisms associated with neuronal guidance, a central problem in developmental neurobiology. Eph receptor tyrosine kinases and their cell surface-bound Ephrin ligands are known to modulate the guidance of many developing neurons in the vertebrate nervous system, but the overlapping expression patterns and promiscuous interactions among multiple ligand-receptor pairs have hindered a functional analyses *in vivo*. As an alternative strategy, I have investigated the role of Ephrin-Eph receptor interactions in the control of neuronal migration within the developing enteric nervous system (ENS) of the moth, *Manduca sexta*. The ENS is formed by a population of approximately 300 neurons (the EP cells) that migrate along a set of identified muscle band pathways on the midgut while avoiding the enteric midline. I have shown that the EP cells express a single class-A Ephrin (MsEphrin; a GPI-linked ligand), which can be detected in their filopodial processes as they explore the midgut surface. Concurrently, the midline interband regions of the midgut (which are inhibitory to migration) express MsEph, the sole Eph receptor homologue in *Manduca*.

To investigate the role of MsEphrin-MsEph interactions in the ENS, I manipulated their interactions during EP cell development using a combination of Fc- and 6His-tagged fusion proteins, and knocked down MsEphrin expression levels using antisense Morpholino oligonucleotides. The results of these experiments suggested that normal MsEphrin-MsEph receptor interactions mediate cell-cell repulsion between the EP cells and the midline cells, a mechanism that restricts migration and outgrowth to the muscle band pathways. In addition, the results from these experiments suggested a novel

role for reverse signaling via a GPI-linked Ephrin ligand in the control of neuronal guidance.

Reverse signaling mediated by the Ephrin-B subclass has been well documented. Ephrin-B ligands have a transmembrane domain and short cytoplasmic domain, which permit direct communication with downstream effectors. In contrast, little is known about the mechanisms associated with Ephrin-A-mediated reverse signaling; their GPI anchorage to the membrane does not provide them direct communication into the cell. Using a candidate approach, I discovered that a *Manduca* Src ortholog (in its active form; phospho-Src) colocalizes with MsEphrin during migration and outgrowth. Pharmacological manipulations to Src in the developing ENS showed that Src activation functions to prevent midline crossing in a manner similar to MsEphrin-dependent reverse signaling. Using single-gut explant assays and high-resolution confocal imaging of manipulated EP cells, I discovered that MsEphrin-mediated reverse signaling promotes the local phosphorylation of Src. Additional experiments combining MsEph-Fc (to overstimulate reverse signaling) and PP2 (to inhibit Src activation) demonstrated that Src phosphorylation is necessary for MsEphrin-mediated reverse signaling in the developing ENS. This investigation represents the first *in vivo* demonstration of a signaling mechanism associated with reverse signaling via a GPI-linked Ephrin ligand. This work provides a platform for further investigations into the molecular and developmental mechanisms related to MsEphrin-MsEph receptor interactions in the developing ENS.

CHAPTER 1

Thesis Introduction

Introduction to the scientific question of neuronal guidance.

During development, neurons must migrate and project their axons over substantial distances before they form synaptic contacts with their target tissues. In order for a nervous system to form appropriately, neurons must reliably interpret the myriad of guidance cues that they encounter along their migratory route. Not surprisingly, defects in neuronal guidance lead to a variety of congenital brain malformations and postnatal neurological impairments. What are the mechanisms that control neuronal guidance? This question was first posed by Santiago Ramon y Cajal after his discovery of neuronal migration and axon outgrowth in 1890[1], and has since been the subject of intense investigation. We now know of a plethora of molecular guidance cues, many of which are ligands that bind receptors on a neuron's cell surface, and often these events induce intracellular signaling that directly modulates cytoskeletal dynamics (Tessier-Lavigne and Goodman, 1996). Guidance factors may either be diffusible ligands that form directional gradients, cell-surface-bound ligands that provide contact-mediated directional information, or extracellular matrix-associated substrate adhesion molecules (Tessier-Lavigne and Goodman, 1996). Over the last three decades, extensive

[1] In his autobiography Recollections Of My Life, Cajal reflects poetically on one of his greatest discoveries: "I had the good fortune to behold for the first time that fantastic ending of the growing axon. In my sections of the three-day chick embryo, this ending appeared as a concentration of protoplasm of conical form, endowed with amoeboid movements. It could be compared to a living battering ram, soft and flexible, which advances, pushing aside mechanically the obstacles which it finds in its way, until it reaches the area of its peripheral distribution. This curious terminal club, I christened the *growth cone*." Cajal SR. 1996, originally published in 1901 as Recuerdos de Mi Vida. Recollections of my life. E. Horne Craigie JC, translator: MIT Press.

information has come from biochemical investigations of the signaling pathways that are modulated by particular guidance factors, while our understanding of how individual guidance factors can alter the behavior of growing neurons has come largely from studies using explanted neurons (*in vitro*). In whole-animal studies, much has also been gleaned from elegant genetic models that use mutagenesis to identify novel guidance factors or transgenesis approaches by which specific factors have been misexpressed. One of the challenges in the field of neuronal guidance is bridging the gap between the signaling mechanisms identified using *in vitro* methods, and the phenotypes observed after manipulations affecting whole animal models *in vivo*. In this thesis, the expression, biological function, and intracellular signaling mechanisms associated with a particular family of guidance molecules (the Ephs and Ephrins) have been investigated *in vivo*, specifically within the context of the developing enteric nervous system (ENS) of the tobacco hornworm *Manduca sexta*.

The *Manduca* ENS as a model system for investigating neuronal guidance.

The *Manduca* ENS is a useful model for analyzing neuronal motility *in vivo* (Copenhaver, 2007). A thorough embryological description of neuronal migration and axon outgrowth in this system is provided in detail in chapters 2, 3 and 4. See chapter 2, figure 1 for a schematic illustration of the developing ENS. Briefly, the ENS is formed largely by a population of neurons (named the "EP cells" because they populate the "enteric plexus") that migrate in a stereotyped manner along preformed muscle band pathways on the midgut (Copenhaver and Taghert, 1989b). After their rapid migration, they project fasciculated bundles of axons along these muscle bands, and subsequently

send defasciculated branches laterally to innervate the musculature of the gut (Copenhaver and Taghert, 1989b).

At the inception of this thesis, several bodies of work had described distinct molecular guidance mechanisms in the ENS. First, it was shown that EP cell migration was regulated by calcium spiking activity within the migratory EP cells, which were coupled to the activity of heterotrimeric G-proteins (Horgan and Copenhaver, 1998; Horgan et al., 1995). Elegant embryological experiments using classical transection and cell transplantation methods also demonstrated that the muscle band pathways were necessary for EP cell motility, and that they likely expressed one or more positive regulatory cues that promoted migration and outgrowth (Copenhaver et al., 1996). Fasciclin II (MsFas II), the *Manduca* homolog of NCAM and OCAM, was subsequently identified as one such factor that promotes migration: homophilic interactions between MsFas II expressed by the EP cells and the muscle bands was shown to be necessary for migration and axon outgrowth (Wright and Copenhaver, 2001; Wright et al., 1999).

However, there remained a variety of mysteries to be solved, even in this simple system. For example, why do the EP cells migrate, for the most part, in an anterior to posterior manner? Is there a diffusible chemoattractant or chemorepellent present? In addition, the EP cells clearly prefer to migrate along the band pathways, but normally do not travel between bands (the "interband" regions) (Copenhaver et al., 1996; Copenhaver and Taghert, 1989b; Wright and Copenhaver, 2001). Given this observation, what local guidance cues, if any, are present that inhibit ectopic growth into the lateral musculature (see chapter 2, figure 1A; e.g. between R1 and R2)? Preliminary studies have suggested that the *Manduca* homolog of the amyloid precursor protein (APPL), which is expressed

by the EP cells (Swanson et al., 2005), may serve as a receptor for unknown ligands on the lateral musculature, inducing a chemorepulsive response in the EP cells by stimulating G-protein-dependent calcium oscillations, as described above (P.F.C. unpublished observations). The thesis presented here poses a second question: what molecular factors are present to inhibit the migratory neurons from crossing the midline?

Introduction to Ephrin-Eph receptor signaling.

In vertebrates, the Eph receptors constitute the largest known family of receptor tyrosine kinases, which are activated by cell-surface bound ligands, the Ephrins. Although the Ephs and Ephrins are expressed in many other tissue types, they are classically known for their roles as guidance factors during the development of the vertebrate nervous system. For reviews, see (Flanagan and Vanderhaeghen, 1998; Kullander and Klein, 2002; Pasquale, 2005; Wilkinson, 2001). The first Eph receptor, EphA1, was first isolated in, an Erythropoietin-producing hepatoma cell line, giving rise to the designation of 'Eph' receptor (Hirai et al., 1987). Since then, there have been 15 additional receptors characterized (Pasquale, 2005), which fall into two subclasses, based on their preferential affinities for Ephrin ligands (see introductory figure 1): EphA receptors generally bind Ephrin-A ligands, which are attached to the plasma membrane via glycosylphosphatidyl inositol (GPI) membrane linkages. Conversely, EphB receptors preferentially recognize Ephrin-B ligands, which are type-1 transmembrane proteins that have a cytoplasmic tail capable of transmitting intracellular signals. The original assignment of these primary receptor classes was based on analyses of the dissociation constants for different receptor and ligand pairs *in vitro* (Flanagan and Vanderhaeghen,

1998). Subsequently, however, cross-talk has been observed between the two receptor classes and the different Ephrin subtypes (Himanen et al., 2004). By contrast, insects appear to express just one Eph receptor and one Ephrin ligand, and therefore present experimental opportunities not possible in vertebrate preparations (summarized below). An interesting debate in this field pertains to the question as to why vertebrates have evolved so many variations of these molecules, many of which overlap both functionally and in their patterns of expression. One plausible argument is that the vast number of receptors and ligands in higher mammals is reflective of the acquisition of higher neurological function (Flanagan and Vanderhaeghen, 1998). However, it is interesting that all vertebrates, ranging from *Xenopus* to humans, encode the same number of Ephs and Ephrins, suggesting that their number does not likely correlate with advances in intellect, but rather a more ancient advance in the overall organization of the nervous system.

Ephrin-Eph receptor interactions in the developing nervous system.

The current body of literature pertaining to Ephrin-Eph receptor interactions in developing nervous systems is extensive, and is therefore beyond a comprehensive review in this chapter. However, a few historical points should be noted as a prelude to a discussion of how a simple insect model system provides a unique opportunity to examine Ephrin-Eph receptor functions *in vivo*.

The first indication that Ephrin-Eph receptor signaling is important in the developing nervous system stemmed from work by Elena Pasquale and colleagues, who showed that Cek5 (now known as EphB2) could be immunolocalized to axonal tracks

within the hippocampus and cerebellum of the developing chick (Pasquale et al., 1992). After this initial discovery, the expression patterns of several other Eph receptors were described on growing axons, suggesting a prominent guidance role for these molecules during nervous system development. Ephrin-A2 and Ephrin-A5 were then shown to be expressed in complementary gradients with their receptors (Cheng et al., 1995; Drescher et al., 1995), and Ephrin-A5 exerted chemorepulsive effects on explanted neurons *in vitro* (Drescher et al., 1995; Zhang et al., 1996). These studies were the first to suggest that Ephrin-Eph receptor interactions might be the long sought-after guidance factors that help establish topographic maps formed by growing axons in particular regions of the brain. As a result, these discoveries led to the postulation of a now-classic model: Ephrin-Eph receptor interactions mediate the organization of retinotectal topographic projections primarily through the chemorepulsive effects of Ephrins (Flanagan and Vanderhaeghen, 1998; Wilkinson, 2001). Ephs and Ephrin are also known to establish topographic maps for vomeronasal projections (Knoll et al., 2001b), and within the hippocampal-septal system (Gao et al., 1996; Zhang et al., 1996).

Besides helping to form topographic projections in the vertebrate nervous system, Ephs and Ephrins have also been implicated in a number of additional processes associated with neural differentiation, and a variety of experimental methods have been developed to investigate their functions. Why, then, develop an insect model? In vertebrates, investigations *in vivo* have been complicated by the overlapping patterns of expression that have been described for multiple Ephs and Ephrins, many of which exhibit promiscuity in their interactions (Davy and Soriano, 2005; Himanen et al., 2004; Poliakov et al., 2004). Not surprisingly, genetically altered mice in which only one Eph

receptor or Ephrin has been eliminated typically have few or no phenotypic defects, and often strong phenotypes have only been observed when at least two or three of these molecules have been disrupted (Mendes et al., 2006; Pfeiffenberger et al., 2006). Consequently, most investigations of particular receptor-ligand interactions in vertebrates have been limited to preparations where a neuron subtype of interest has been explanted and manipulated *in vitro*. Moreover, the complexity of vertebrate systems does not readily permit an analysis of the signaling mechanisms controlling cellular behavior *in vivo*, because of the difficulty in achieving single-cell or sub-cellular resolution in an intact brain.

In contrast, the relative simplicity of several different invertebrate preparations has facilitated mechanistic investigations into the role of specific Ephrin-Eph receptor interactions *in vivo*. For example, it was recently shown that in the sea squirt *Ciona intestinalis*, intercellular contacts mediated by Ephrin-Eph interactions normally downregulate ERK signaling as a requirement for asymmetric cell division (Picco et al., 2007; Shi and Levine, 2008). In the honeybee *Apis mellifera*, *Am*Eph and *Am*Ephrin are differentially localized throughout the brain and may function in olfactory learning and memory (Vidovic et al., 2007). In *Drosophila*, DEph and DEphrin may regulate the formation of commissures within the nerve cord (Bossing and Brand, 2002; Scully et al., 1999) and axonal pathfinding during mushroom body formation (Boyle et al., 2006). This thesis employs the simplicity of Ephrin-Eph receptor interactions in the *Manduca* ENS, a system where only one Eph receptor (MsEph) and one Ephrin ligand (MsEphrin; GPI-linked) is expressed. This model thus provides the opportunity to examine how one Ephrin-Eph receptor pair contributes to neuronal guidance in a normal developmental

context.

Ephrin-Eph receptor bidirectional signaling.

An unusual feature of Ephrin-Eph receptor interactions is that their activation can be "bidirectional," where both the receptor and ligand transduce intracellular signals (Fig. 1). Conventional forward signaling is induced when Ephrin ligands stimulate Eph receptors on adjacent cells, leading to the autophosphorylation of several tyrosine residues within the cytoplasmic domain of the Eph receptors. This event leads to the recruitment of a variety of downstream effectors, many of which possess SH2 and SH3 protein-protein interaction domains (Kullander and Klein, 2002). Forward signaling via Eph receptors mediates a diversity of cellular responses (Pasquale, 2005), many of which involve the modulation of cytoskeletal dynamics that regulate intercellular adhesion (Kullander and Klein, 2002).

Besides forward signaling, *reverse* signaling also can occur, by which Eph receptors activate Ephrin ligands as a result of cell-cell contact. Ephrin-B ligands possess a small cytoplasmic domain (Fig. 1) that can be phosphorylated in response to Eph binding (Bruckner et al., 1997), which in turn can lead to the propagation of a variety of intracellular signals that may regulate a variety of responses via modulation of the cytoskeleton, including repulsion, directed migration, or axon steering (Bruckner et al., 1999; Cowan and Henkemeyer, 2001; Lin et al., 1999; Lu et al., 2001).

Surprisingly, reverse signaling can also occur via GPI-linked Ephrins, which was surprising, as these ligands have no direct contact with the cytoplasm (see Fig. 1), and thus cannot transduce reverse signals on their own. Several reports have demonstrated

that the activation of several cytoplasmic phosphoproteins occurs downstream of Ephrin-A reverse signaling (Davy et al., 1999; Huai and Drescher, 2001), although these findings were limited to manipulations of fibroblasts *in vitro*. Several genetic models have since demonstrated the importance of Ephrin-A reverse signaling in neurogenesis (Holmberg et al., 2005), axon guidance (Knoll and Drescher, 2002), and insulin secretion (Konstantinova et al., 2007), but the signaling mechanisms in these models remain poorly understood. The final component of this thesis investigates the signaling factors that are necessary *in vivo* for reverse signaling mediated by a GPI-linked Ephrin.

The reconciliation of high-affinity adhesion and cell-cell repulsion.

An intriguing paradox of Ephrin-Eph receptor interactions is that the high-affinity and multivalent ligand-receptor complexes that are formed upon cell-cell contact often result not in adhesion, but repulsion. In general, an Eph receptor and Ephrin ligand bind to each other with a dissociation constant around 1 nM (Flanagan and Vanderhaeghen, 1998); thus, additional mechanisms must be present to clear the receptor-ligand complex as a prerequisite for repulsion. Two distinguishable mechanisms were recently proposed that pertain to each subclass of Ephrin ligand. For the Ephrin-A subclass, a proteolysis mechanism may be used to shed Ephrin-A ectodomains from the membrane upon engagement with EphA receptors (Hattori et al., 2000). The metalloprotease Kuzbanian (ADAM-10), which has been implicated previously in Delta-Notch processing (Pan and Rubin, 1997), was shown to induce efficient shedding of Ephrin-A2 from transfected NIH-3T3 cells upon engagement with

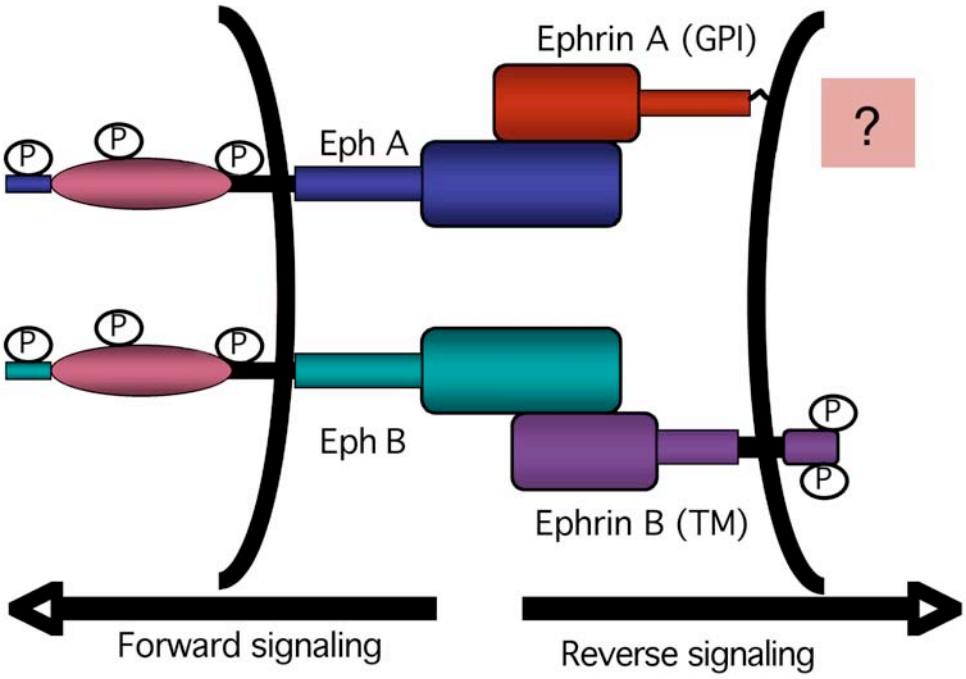

Figure 1.1. Schematic representation of Eph receptors and Ephrin ligands. EphA receptors (blue) are mainly activated by GPI-linked Ephrin-A ligands (red). EphB receptors (turquoise) are mainly activated by Ephrin-B ligands (purple), which contain a transmembrane (TM) domain and short cytoplasmic tail. Forward signaling results in tyrosine phosphorylation of the cytoplasmic domain of Eph receptors, which leads to the recruitment of downstream effectors. Reverse signaling leads to downstream signaling via tyrosine phosphorylation of the cytoplasmic tail of Ephrin-B ligands, and also by Ephrin-A ligands, but by relatively unknown mechanisms (see "?"). Forward and reverse signaling requires cell-cell contact. Figure adapted from (Kullander and Klein, 2002).

EphA3-expressing cells. This cleavage event was necessary for the subsequent repulsive response normally elicited in the neurons; otherwise intercellular adhesion was maintained (Hattori et al., 2000). For the Ephrin-B subclass, an entirely different, yet equally intriguing, mechanism of contact-mediated repulsion has been reported (Marston et al., 2003; Zimmer et al., 2003). In tissue culture experiments, interactions between cells that expressed either EphB2 or Ephrin-B1 generated a trans-endocytosis response, by which intact receptor-ligand complexes were rapidly engulfed into each adjoining cell (Zimmer et al., 2003). This event effectively relieved the transient adhesion mediated by

Ephrin-B1-EphB2 interactions between adjacent cells, thereby facilitating their repulsion. In addition, signaling activity on the C-terminal domains of both the ligand and receptor must be intact in order for endocytosis to occur, suggesting that this mechanism may be limited to the Ephrin-B subclass. How does the reconciliation of adhesion and repulsion occur *in vivo*? Chapter 5 of this thesis discusses methods by which the insect ENS might provide a platform for experimentally testing this question in a biologically relevant context.

Ephrin-Eph receptor interactions in cancer and human brain disease.

Interestingly, every Eph receptor and Ephrin in vertebrates has been identified as being misexpressed or dysregulated in some form of tumor (Heroult et al., 2006). For example, EphA2 is upregulated in tumors of the prostrate, lung, colon, skin and breast, with highest expression levels correlating with progression into late stages of cancer and poor patient prognosis (Heroult et al., 2006; Ogawa et al., 2000). When overexpressed, EphA2 promotes malignant transformation of mammary epithelial cells *in vitro* (Zelinski et al., 2001). Another well-documented example is EphB2, which is upregulated in tumors of the liver, gastrointestinal tract, ovary, lung and kidney (Heroult et al., 2006). The presence of Ephs and Ephrins in tumor tissues is not entirely surprising, in part because they are known to have prominent roles in cell migration and adhesion, but also because they are intimately involved in the regulation of both cell proliferation (Pasquale, 2005) and neovascularization during development and adult life (Adams et al., 1999; Wang et al., 1998). As the metastatic process often recapitulates developmental events (Heroult et al., 2006), understanding the normal signaling mechanisms by which Ephrin-

Eph receptor interactions control cell proliferation, growth and differentiation is essential for the identification of appropriate molecular targets for therapeutic intervention.

Given the importance of Ephs and Ephrins in normal brain development, it is not surprising that mutations in the genes encoding them have also been linked to a spectrum of congenital brain defects. In particular, defects in neuronal guidance resulting from misregulated Ephrin-Eph receptor interactions have been implicated in a number of different brain malformations. For example, the guidance of contralateral axonal projections across the midline of the brain is modulated by a number of Ephrin-Eph receptor combinations, and in transgenic mice, mutations in several of these genes result in agenesis of the corpus callosum (ACC) (Hu et al., 2003; Mendes et al., 2006). Likewise, in humans, defects in particular Ephrins have now been implicated in several syndromes that present with ACC. Ephrin-B1 has been genetically mapped in patients with FG syndrome (Mendes et al., 2006), while mutations in this gene have been directly linked to craniofrontonasal syndrome in both mice and humans, a disorder that perturbs cranial neural crest development as well as callosal formation (Twigg et al., 2006; Wieland et al., 2004; Wieland et al., 2005).

Ephrins and Eph receptors also participate in injury responses within the CNS, and play important roles in a number of neurological diseases. Misregulation of several different Ephrins and Ephs has been detected following lesions to the spinal cord and optic nerve, while their upregulation in astrocytes and oligodendrocytes may promote glial scarring and inhibit axonal regeneration (Goldshmit et al., 2006). Intriguingly, EphA receptors and their Ephrin-A ligands are also upregulated in white matter lesions associated with Multiple Sclerosis, where they may exacerbate neurodegeneration

(Goldshmit et al., 2006; Sobel, 2005). Finally, reverse signaling via GPI-linked Ephrins has recently been suggested to enhance kindling during epileptic seizures by promoting ectopic neural outgrowth (Xu et al., 2003). Despite the increased awareness of the involvement of Ephrins and Eph receptors in both normal and abnormal aspects of neurological function, the molecular mechanisms underlying these processes remain largely unexplored.

Thesis overview.

In this thesis, I describe the results of my investigations into the role of a specific Ephrin (MsEphrin) and its cognate Eph receptor (MsEph) in the control of neuronal migration within the developing ENS of *Manduca*. I also describe my discovery of a molecular mechanism by which reverse signaling through this GPI-linked Ephrin controls neuronal motility in vivo. In chapter 2, the developmental expression patterns of MsEphrin and MsEph mRNA and protein within the developing ENS are first described. Briefly, MsEphrin and MsEph receptor expression is complementary: MsEphrin is expressed solely by the EP cells during phases of migration and axon outgrowth, while MsEph receptor expression is restricted to the midline muscle cells. In chapter 3, I then present experimental evidence that supports a model whereby repulsive interactions between MsEphrin and MsEph are required to prevent the EP cells from aberrantly crossing the midline. The results presented in this chapter strongly argue that *reverse* signaling via MsEphrin rather than *forward* signaling via MsEph is the primary mechanism that facilitates cell-cell repulsion in this system. In chapter 4, I present evidence that reverse signaling via MsEphrin leads to the phosphorylation of a *Manduca*

Src family kinase. In particular, I show that this phosphorylation event is necessary for repulsion of the EP cells and their processes at the midline, and thus critical for appropriate formation of the ENS. In the final chapter (chapter 5), possible future directions are proposed for this project, along with preliminary data that may be useful to future investigators.

CHAPTER 2

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

Journal of Comparative Neurology. 2007 May 10;502(2):175-91.

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Tracy Swanson provided the Northern Blots in figures 2B and 6A. Thomas Proctor provided the transmission electron micrograph in Figure 1H. Alan Nighorn provided DNA constructs that were used to generate the Fc-tagged fusion proteins (MsEph-Fc and MsEphrin-Fc; used in figure 7). Philip Copenhaver provided the scanning electron micrographs in Figure 1D-F (see also Copenhaver et al., 1989b).

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.

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 (Kullander and Klein, 2002; Wilkinson, 2001). Eph receptors can be categorized into A or B subgroups, based on their preferential affinities for different subsets of Ephrin ligands. EphA receptors generally bind Ephrin-A ligands, which are distinguished by their glycosyl phosphatidylinositol (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 (Cooke et al., 2001; Cooke et al., 2005; Mellitzer et al., 1999) 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 (Davy and

Soriano, 2005; Himanen et al., 2004; Poliakov et al., 2004). 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 (Bossing and Brand, 2002; Scully et al., 1999), where they may help promote the segregation of axons during commissure formation (Bossing and Brand, 2002; Scully et al., 1999) 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 pre-formed 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 et al., 1996; Copenhaver and Taghert, 1989a; Copenhaver and Taghert, 1989b). 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 25ºC. 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 $CaCl_2$; 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 μm) were then collected on polylysine-coated glass microscope slides, cleared in SafeClear (Fisher Scientific, Pittsburg, 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% OsO_4, 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-^{32}P]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 65ºC for the MsEph receptor and 80ºC 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 60ºC. 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% NaN_3). 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 4ºC 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 -20º 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 -20º 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 μg/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, 1989b; 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, 1989a). 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

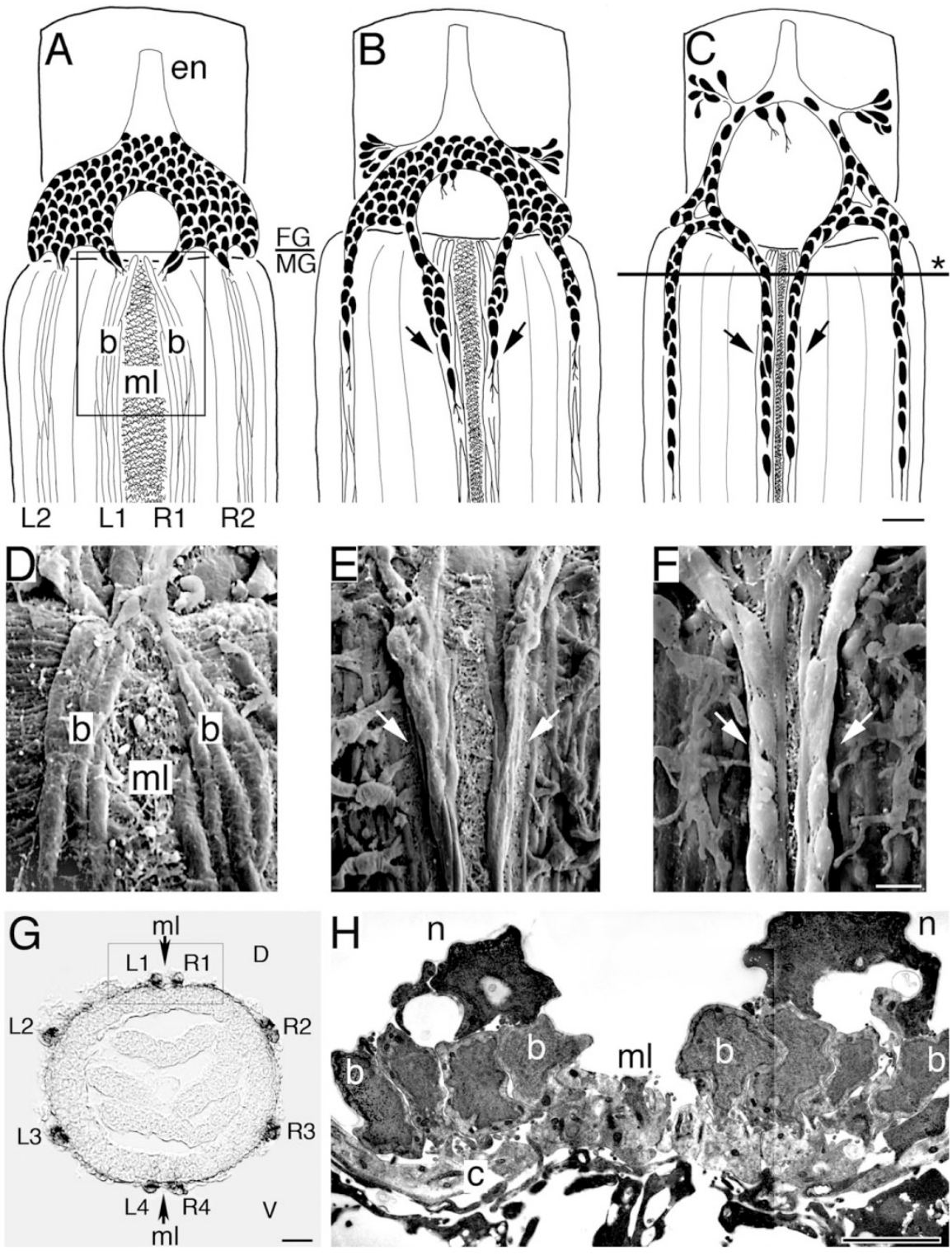
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Figure 1.

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.

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 (Dephrin), 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. peudoobscura*),

mosquitoes (*Anopheles gambiae*), honeybees (*Apis mellifera*), and silkmoths (*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 ^{32}P-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

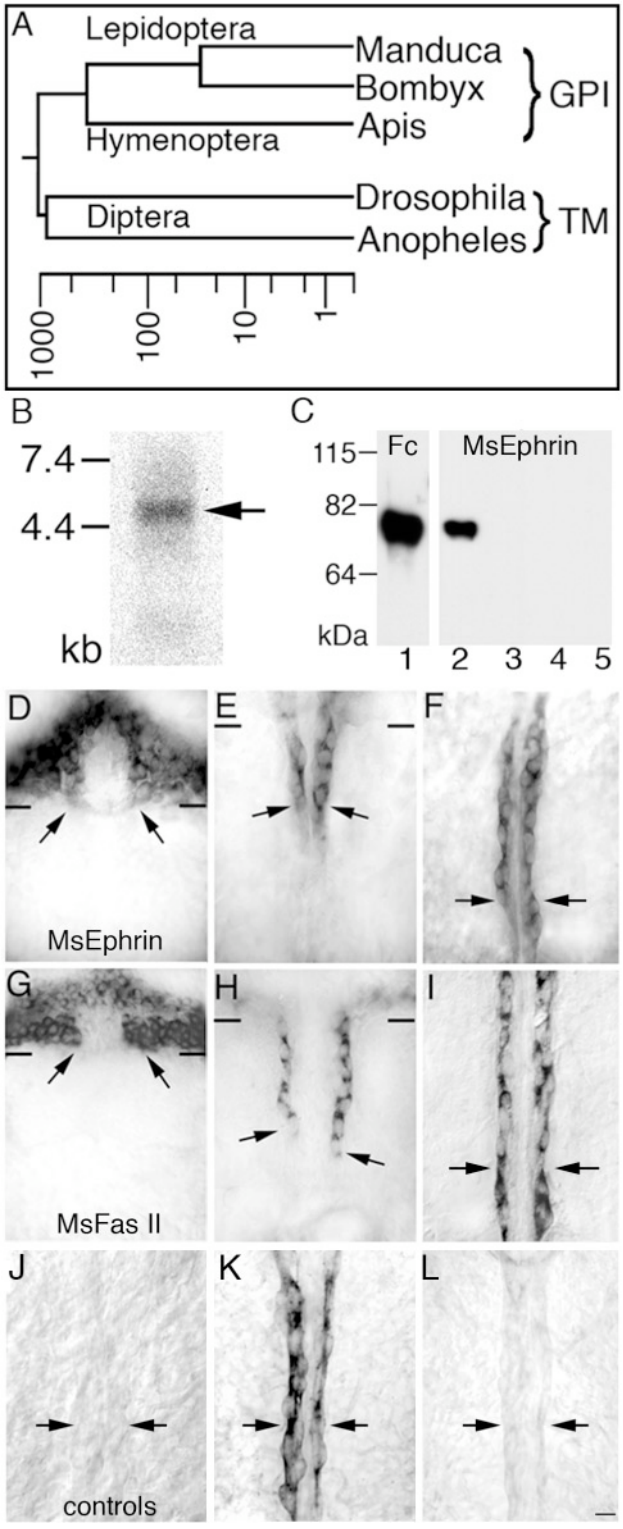
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Figure 2.

Figure 2. A single isoform of MsEphrin is expressed by the migratory EP cells. (A) Dendrograph 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 μm. (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 μm.

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 and Copenhaver, 2000; Wright et al., 1999). 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 and Copenhaver, 2000; Wright et al., 1999). In contrast, MsEphrin expression was restricted to the EP cells. Application 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).

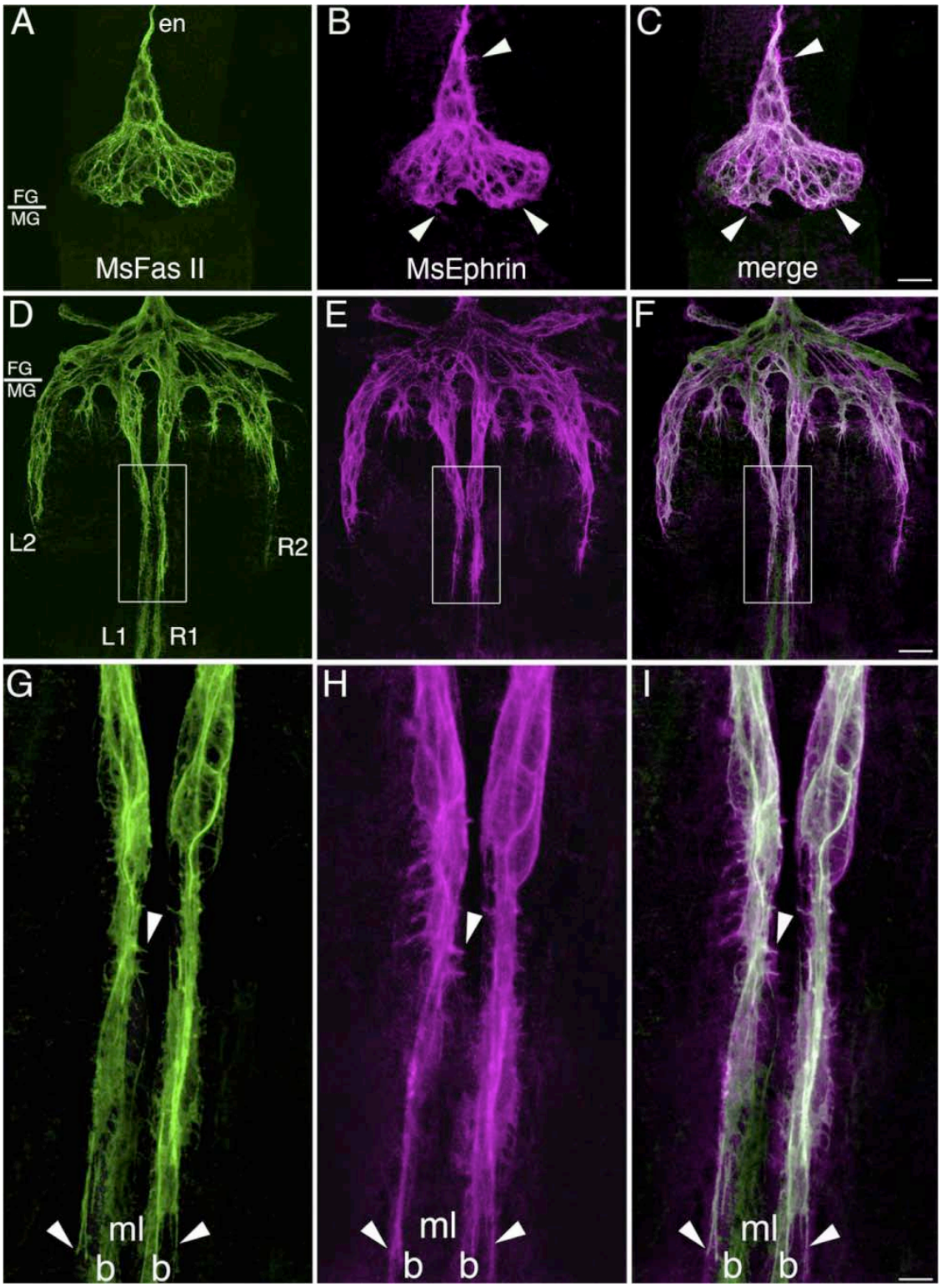
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Figure 3.

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

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).

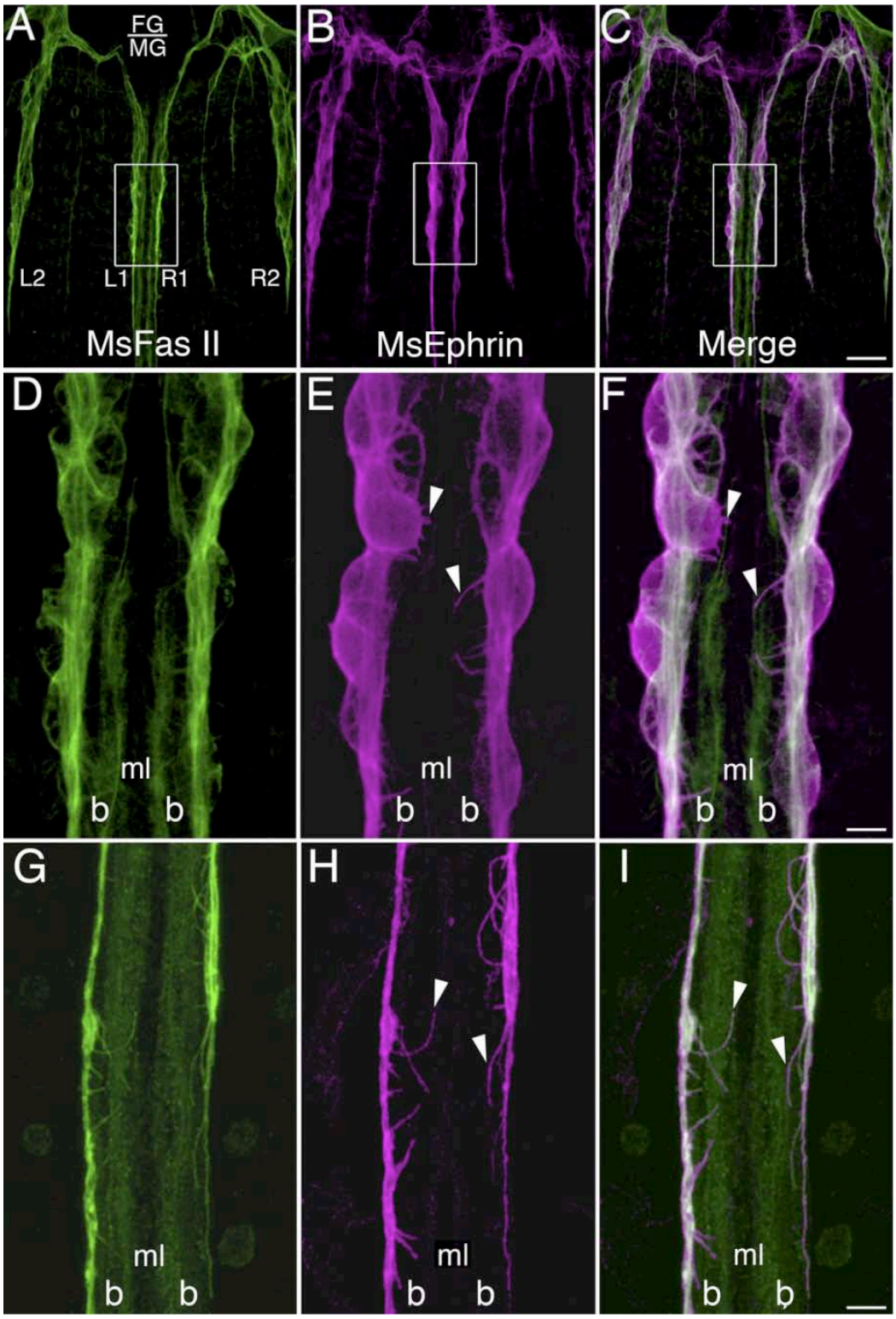
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Figure 4.

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 μm in A-C and 10 μm in D-I. FG/MG = foregut/midgut boundary.

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

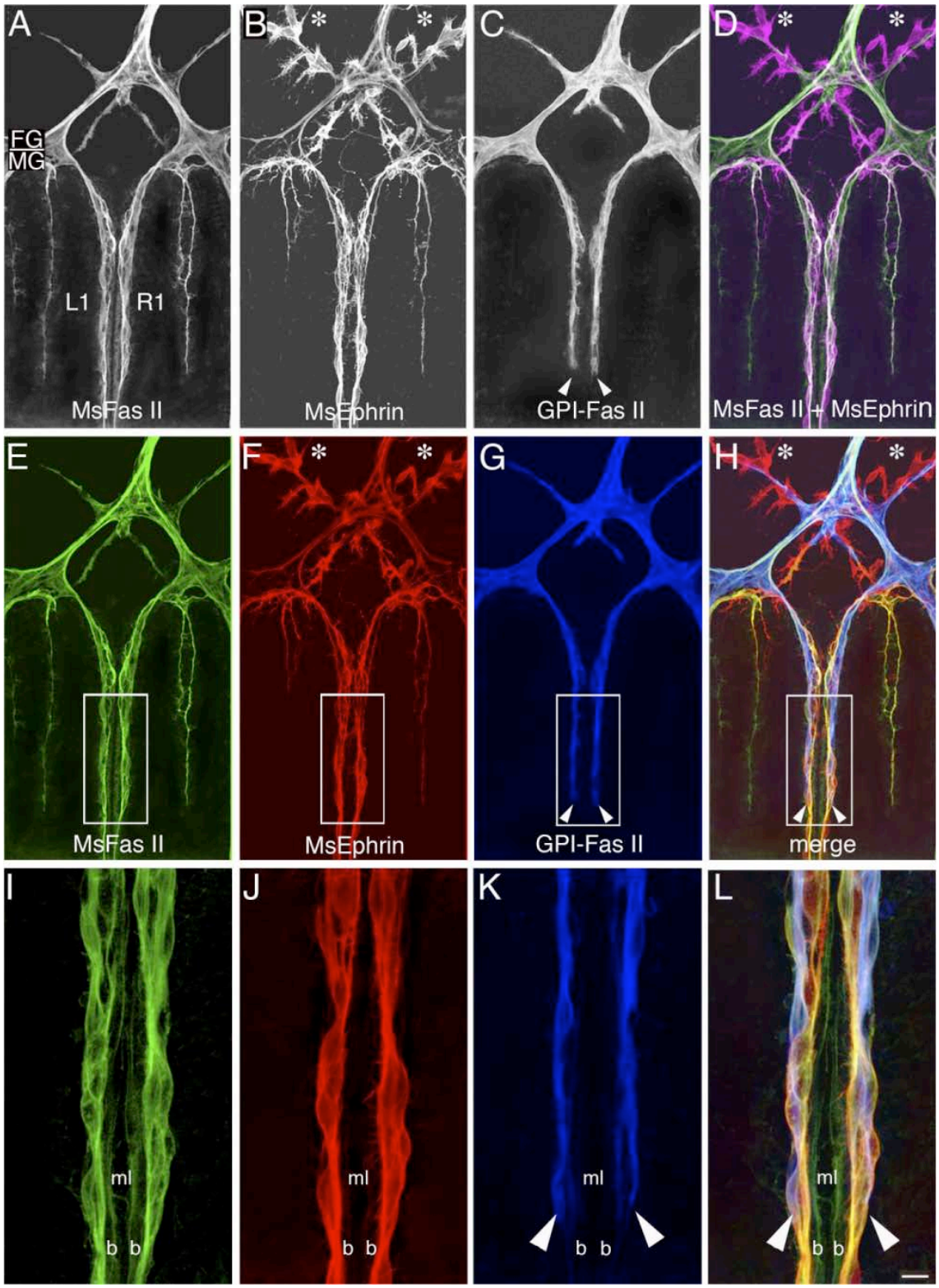
Once the EP cells have achieved their mature positions on the foregut and midgut and have begun to extend processes onto the adjacent visceral musculature (Copenhaver and Taghert, 1989a; Copenhaver and Taghert, 1989b), a subsequent wave of migratory glial cells ensheathes the major branches of the enteric 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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F
MsEphrin
G
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Figure 5.

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.

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-

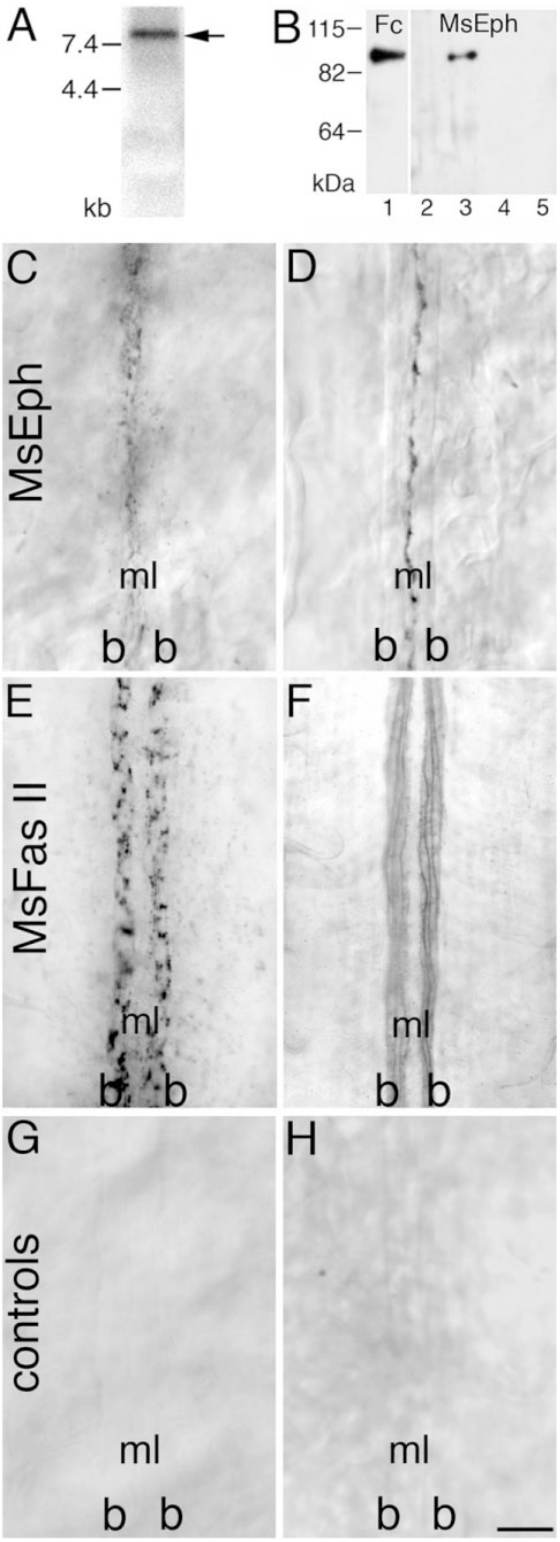

Figure 6. A single mRNA species encoding the MsEph receptor is expressed by the midline interband cells. (A) Northern Blot of total RNA from embryos labeled with ^{32}P-labeled antisense riboprobes specific for MsEph receptor mRNA reveals a single band at ~8.0 kb. (B) Immunoblot of Fc fusion proteins 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 μm.

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

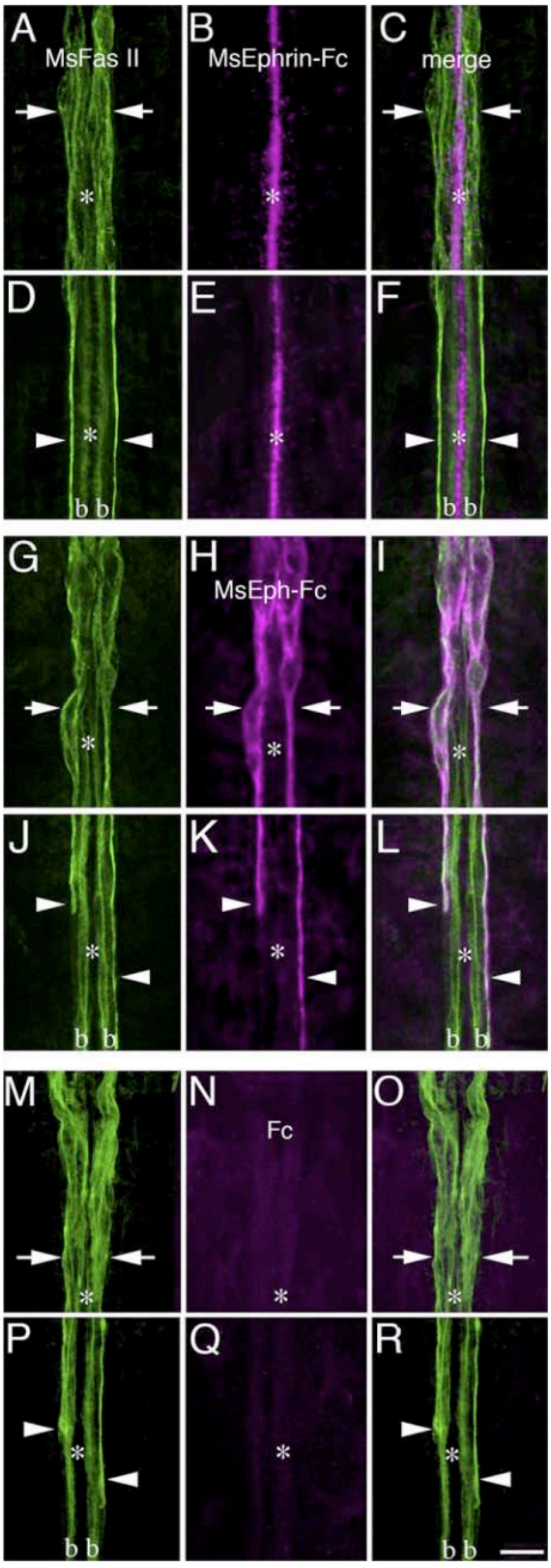

Figure 7. Fc-fusion proteins of MsEphrin and the MsEph receptor label the midline cells and EP cells, respectively. (A-F) A 62% embryo that was incubated with MsEphrin-Fc, fixed, and then double-immunostained with anti-Fc (magenta) and anti-MsFas II antibodies (green). Panels A-C show the anterior midgut 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 μm.

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 μg/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

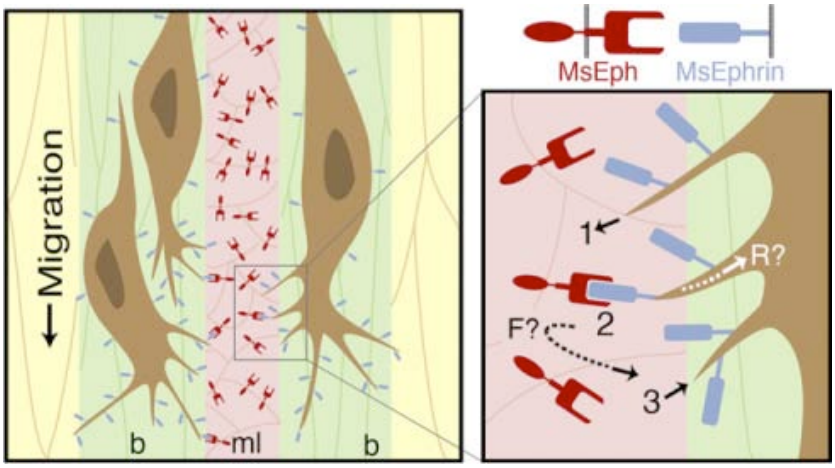

Figure 8. A model for the regulation of EP cell migration at the ENS midline by MsEphrin-MsEph receptor interactions. Left panel: migrating EP cells extend filopodia enriched in MsEphrin (blue) along their muscle band pathways (**b**; green), as well as onto the adjacent midline cells (**ml**; pink) 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.

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 and Copenhaver, 2000; Wright

et al., 1999). 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 and Copenhaver, 2000; Wright et al., 1999). 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 (Kullander and Klein, 2002; Wilkinson, 2001), 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 (Knoll et al., 2001a; Krull et al., 1997; Wang and Anderson, 1997), 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 (Davy et al., 2004; Kullander and Klein, 2002). Reverse signaling through GPI-linked Ephrins (like MsEphrin) has also been implicated in several instances (Holmberg et al., 2005; Knoll

and Drescher, 2002), 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 et al., 1996; Copenhaver and Taghert, 1989a; Copenhaver and Taghert, 1989b), 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 (Burns, 2005; Le Douarin and Kalcheim, 1999), while in insects, neural precursors of the ENS invaginate from neurogenic placodes in the ectodermal foregut (Copenhaver and Taghert, 1990; Copenhaver and Taghert, 1991; Ganfornina et al., 1996; Hartenstein et al., 1994). 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 (Anderson et al., 2006; Wright and Copenhaver, 2000; Yoneda et al., 2001) 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. (Davy and Soriano, 2005; Krull et al., 1997; Wang and Anderson, 1997). 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 McLaughlin, 2005; O'Leary and Wilkinson, 1999). 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 (Davy and Soriano, 2005; Himanen et al., 2004; Poliakov et al., 2004). 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 complementary 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.

ACKNOWLEDGEMENTS

We thank Drs. Megumi Kaneko and Alan Nighorn for generously sharing their MsEphrin and MsEph receptor constructs and advice, and Dr. Stephanie Kaech-Petrie (in the Live Cell Imaging Facility, Center for Research on Occupational and Environmental Toxicology) for her excellent instruction and assistance with confocal imaging. We also thank Drs. Doris Kretzschmar, David Morton, the members of their laboratories, and Mr. Todd Vogt in our laboratory for their insightful discussions. Dr. Tiffani Howard provided the artwork for figure 8 (tiffani@howardink.com). We also thank Drs. Larry Sherman, David Morton, Michael Forte, and Doris Kretzschmar for critical evaluations of this manuscript.

CHAPTER 2

Reverse signaling via a GPI-linked Ephrin prevents midline crossing by migratory neurons during embryonic development in *Manduca*.

Journal of Neuroscience. April 9, 2008, 28(15):3846-3860

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Jacqueline Wirz provided analytical ultracentrifugation data (not shown).

ABSTRACT

We have investigated whether reverse signaling via a GPI-linked Ephrin controls the behavior of migratory neurons *in vivo*. During the formation of the enteric nervous system (ENS) in the moth *Manduca*, ~300 neurons (EP cells) migrate onto the midgut via bilaterally paired muscle bands but avoid adjacent midline regions. As they migrate, the EP cells express a single Ephrin ligand (MsEphrin; a GPI-linked ligand), while the midline cells express the corresponding Eph receptor (MsEph). Blocking endogenous MsEphrin-MsEph receptor interactions in cultured embryos resulted in aberrant midline crossing by the neurons and their processes. In contrast, activating endogenous MsEphrin on the EP cells with dimeric MsEph-Fc constructs inhibited their migration and outgrowth, supporting a role for MsEphrin-dependent reverse signaling in this system. In short-term cultures, blocking endogenous MsEph receptors allowed filopodia from the neurons' growth cones to invade the midline, whereas activating neuronal MsEphrin led to filopodial retraction. MsEphrin-dependent signaling may therefore guide the migratory neurons in the ENS by restricting the orientation of their leading processes. Knocking down MsEphrin expression in the EP cells with morpholino antisense oligonucleotides also induced aberrant midline crossing, consistent with the effects of blocking endogenous MsEphrin-MsEph interactions. Unexpectedly, this treatment enhanced the overall extent of migration, indicating that MsEphrin-dependent signaling may also modulate the general motility of the EP cells. These results demonstrate that MsEphrin-MsEph receptor interactions normally prevent midline crossing by migratory neurons within the developing ENS, an effect that is most likely mediated by reverse signaling through this GPI-linked Ephrin ligand.

INTRODUCTION

The formation of the nervous system in many organisms requires the precise migration of neurons along pathways that are delineated by a combination of stimulatory and inhibitory guidance cues. The Eph family of receptor tyrosine kinases and their Ephrin ligands comprise a large group of membrane-associated proteins that can elicit either attractive or repulsive responses, depending on the developmental context (Pasquale, 2005; Wilkinson, 2001). Originally discovered for their role in establishing topographic projections within the retinotectal system (Cheng et al., 1995; Connor et al., 1998; Drescher et al., 1995), Ephrin-Eph interactions have been shown to define spatial gradients and discrete boundaries in many regions of the nervous system and other tissues (Dearborn et al., 2002; Mohamed and Chin-Sang, 2006; Palmer and Klein, 2003; Pasquale, 2005). In vertebrates, 16 different Eph receptors can be grouped by their ligand specificities: EphA receptors preferentially bind GPI-linked (type-A) Ephrins, while EphB receptors preferentially bind transmembrane (type-B) Ephrins (Kullander and Klein, 2002; Pasquale, 2005). Conventional activation of Eph receptors by Ephrin ligands ("forward" signaling) is well-established, but Eph receptors can also promote "reverse" signaling by stimulating Ephrin-dependent responses (Davy and Soriano, 2005; Kullander and Klein, 2002; Murai and Pasquale, 2003). Reverse signaling through type-B Ephrins is often mediated by Src family kinases (SFKs), although SFK-independent signaling has also been described (Cowan and Henkemeyer, 2001; Segura et al., 2007; Wilkinson, 2001). Reverse signaling through type-A Ephrins may similarly involve SFKs or other kinases, albeit via mechanisms that are less well understood (Davy and Soriano, 2005; Holmberg et al., 2005; Knoll and Drescher, 2002).

However, deciphering the role of particular Ephrin-Eph interactions in vertebrates has been complicated by overlapping expression patterns of multiple ligands and receptors in many tissues, and by promiscuous interactions among different ligand-receptor combinations (Davy and Soriano, 2005; Himanen et al., 2004; Marquardt et al., 2005; Poliakov et al., 2004). In contrast, insects typically express only a single Ephrin isoform and one Eph receptor (Bossing and Brand, 2002; Dearborn et al., 2002; Kaneko and Nighorn, 2003; Vidovic et al., 2006). In the tobacco hornworm, *Manduca sexta*, the sole Ephrin ligand (MsEphrin) is a GPI-linked protein that interacts with its receptor (MsEph) in a variety of contexts. In the antennal lobe, complementary distributions of MsEphrin and MsEph receptors promote axonal sorting to specific olfactory glomeruli (Kaneko and Nighorn, 2003), while in the enteric nervous system (ENS), their patterns of expression suggest a role in regulating neuronal migration (Coate et al., 2007). During ENS development in *Manduca*, ~300 neurons (EP cells) must migrate onto the midgut along pre-formed muscle bands without crossing the enteric midline (Copenhaver, 2007; Copenhaver and Taghert, 1989b). MsEphrin is expressed by the neurons and their leading processes, while MsEph receptors are restricted to midline cells (Coate et al., 2007). Using a variety of methods to manipulate endogenous MsEphrin-MsEph interactions in cultured embryos, we have now shown that midline MsEph receptors establish a repulsive molecular boundary that prevents the MsEphrin-expressing EP cells from crossing these regions. In addition, we present evidence that reverse signaling via this GPI-linked ligand regulates neuronal guidance in the developing ENS.

MATERIALS AND METHODS

Animals and whole mount immunohistochemistry.

Synchronized groups of *Manduca sexta* embryos were collected from an in-house breeding colony and staged according to tables of internal and external developmental markers (at 25^{o}C, 1 hr = 1% of development). Dissections were performed in defined saline, as previously described (140 mM NaCl, 5 mM KCl, 28 mM glucose, 40 mM $CaCl_2$, and 5 mM HEPES; pH 7.4; (Coate et al., 2007; Copenhaver and Taghert, 1989b). Unless otherwise indicated, chemicals were purchased from Sigma-Aldrich (St. Louis, MO). For all primary antibodies except anti-MsEphrin, embryos were fixed for 1 hr in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) in PBS, pH 7.4. After rinsing in PBS, preparations were incubated in blocking solution (PBS plus 0.6% Triton X-100, 10% normal serum, and 0.1% Sodium Azide), and primary antibodies (also diluted in blocking solution) were applied for 2 hr at room temperature (RT) or overnight at 4°C. Antibodies were used in the following concentrations: anti-pan-MsFas II: 1:20,000 (monoclonal C3, against the extracellular domain of MsFas II; (Wright et al., 1999), anti-transmembrane MsFas II: 1:1,000 (TM-MsFas II; (Wright and Copenhaver, 2000); monoclonal antibody 4E11 (a marker for the gut midline cells): 1:2500; goat anti-Fc, 1:200 (Jackson ImmunoResearch, West Grove, PA); and goat-anti-Fluorescein isothiocyanate (FITC): 1:100 (Jackson ImmunoResearch). After incubation with primary antibodies, preparations were rinsed for 2 hr and incubated with fluorochrome-conjugated secondary antibodies for 1-2 hr at RT or overnight at 4°C, then rinsed and mounted in SlowFade Gold (Molecular Probes; Eugene, OR). Alexa Fluor-conjugated secondary antibodies (1:1,000) were obtained from Molecular Probes; Cy3-conjugated secondaries

(1:400) were from Jackson ImmunoResearch. For anti-MsEphrin immunostaining, unfixed embryos were incubated for 90 min in chicken anti-MsEphrin (1:100; after (Coate et al., 2007), then rinsed extensively before fixation with 4% paraformaldehyde for 1 hr. The preparations were then incubated with additional primary and secondary antibodies, as described above.

Previously, we developed an anti-peptide antiserum against the MsEph receptor, which revealed that midline interband cells of the midgut are the only cells associated with the developing ENS that express MsEph (Coate et al., 2007). However, because this antiserum proved unsuitable for triple immunolabeling experiments (described below), we used an affinity-purified antibody that recognizes an evolutionarily conserved epitope shared by vertebrate EphB2 and MsEph, generously provided by Dr. M. E. Greenberg and colleagues (at 1:200; (Dalva et al., 2000). As with our original anti-MsEph antiserum, the anti-EphB2 antibody labeled only the midline interband cells in the developing ENS (Supp. Fig 1A). The specificity of this antibody for MsEph in *Manduca* was verified by pre-adsorbing an aliquot against its peptide epitope (also provided by Dr. Greenberg), at a 10:1 molar ratio (peptide:antibody) for 4 hr at RT. This pre-treatment eliminated all immunoreactive staining in *Manduca* embryos (Supp. Fig 1B). In immunoblots of pupal brain lysates (stage P3), the anti-EphB2 antibody labeled a single ~115 kDa band, corresponding to the predicted size of endogenous MsEph (112 kDa; Supp. Fig 1C, lane 1); this band was also eliminated by pre-adsorption of the antibody against its peptide epitope (Supp. Fig. 1C, lane 2). Similar controls for our anti-MsEphrin and MsFas II antibodies have been previously documented (Coate et al., 2007; Wright and Copenhaver, 2000).

Fusion protein preparation and *in vivo* binding assays.

MsEphrin-Fc and MsEph-Fc fusion proteins were generated using published methods (Coate et al., 2007; Kaneko and Nighorn, 2003) and used to target endogenous MsEphrin or MsEph receptors in cultured embryos. To generate MsEphrin-6His and MsEph-6His fusion proteins, 293-EBNA cells were stably transfected with the pCEP4 expression vector (Invitrogen, Carlsbad, CA) containing the sequence for the extracellular domain of either MsEphrin or MsEph, in-frame with a C-terminal 6X Histidine tag (6His). Stable cell lines expressing the fusion proteins were maintained in standard growth medium (DMEM, 10% fetal bovine serum) plus 250 μg/ml G418 and 300 μg/ml Hygromycin B; protein expression levels were monitored in immunoblots using an anti-6His monoclonal antibody (1:5000; Clontech, Mountain View, CA). After sufficient expansion of the cultures, the growth medium was replaced with Opti-MEM (Invitrogen) for an additional 7 days. Secreted 6His-tagged proteins were then purified and concentrated from the medium with cobalt-conjugated Sepharose beads, using Talon resin from Clontech or His-Select Cobalt Affinity resin from Sigma; both resins provided equivalent yields.

Analysis of fusion protein dimerization/oligomerization.

20 ng of each Fc- and 6His-tagged fusion protein were diluted in Laemmli buffer (Laemmli, 1970) with or without β-Mercaptoethanol (βME; 5%), then separated on 4-12% gradient gels (Criterion; Bio-Rad, Hercules, CA) and transferred to nitrocellulose membranes. The immunoblots were then labeled with either anti-Fc or anti-6His antibodies to determine the relative abundance of monomers versus dimers and oligomers

in the samples, based on their predicted sizes. Alternatively, concentrated samples of each fusion protein were subjected to analytical ultracentrifugation, following published methods (Frank et al., 2001; Kobayashi et al., 2007). Briefly, each protein sample was dialyzed against PBS (pH 7.4) plus 150 mM NaCl and concentrated to 0.3-0.5 mg/ml. Sedimentation equilibrium measurements were performed in double sector cells on a Beckman XLA analytical ultracentrifuge at 4 or 20^{o}C, using a rotor speed of 15,000 rpm. Concentrations were monitored at 230 nm as a function of radial distance to determine molecular masses (van Holde, 1985); data were analyzed by non-linear least squares fitting (Scientist, Micromath, St. Louis, MO).

In vivo manipulations of cultured embryos.

Staged embryos were isolated before the onset of EP cell migration (at 52-53% of development) in a customized culture medium (Wright et al., 1999). Embryos were restrained in Sylgard-coated chambers, and the developing ENS was exposed by a small incision in the dorsal body wall. The ENS was then treated either with control culture medium or medium containing one of the epitope-tagged fusion proteins (at 0.1-100 μg/ml). Similar concentrations of human Fc (Jackson ImmunoResearch) were used as an additional control. For some experiments, Fc proteins were pre-clustered for 30 min with anti-human Fc antibodies (Jackson ImmunoResearch) at a 5:1 molar ratio of antibody:Fc protein (after (Davis et al., 1994; Kaneko and Nighorn, 2003). Following application of the fusion proteins, embryos were cultured at 28°C for 24 hr (through the period of EP cell migration and axon outgrowth). At the end of each experiment, the preparations were re-dissected to expose the ENS and immunostained with anti-MsFas II, using the

ABC HRP kit from Vector Laboratories (Burlingame, CA). This procedure allowed the EP cells and their processes to be visualized unambiguously (Coate et al., 2007; Horgan et al., 1995; Wright and Copenhaver, 2000). The extent of EP cell migration, axon outgrowth, and number of midline crossover events was then analyzed using photomicrographic and *camera lucida* techniques (Wright and Copenhaver, 2000; Wright et al., 1999). Statistical analyses were performed using Students' 2-tailed t-test to compare means. Because the frequency of crossover events best fit a Poisson distribution, the Wilcox Signed Rank test was used to calculate significant differences between cumulative frequencies in the different groups. Each experiment was performed at least 3 times.

Filopodial orientation assays.

Embryos were opened in culture at 60% of development (when the EP cells transition from migration to axon outgrowth), and treated with control Fc, MsEph-Fc, or MsEphrin-Fc proteins at 20 μg/ml in defined saline (Horgan and Copenhaver, 1998). This concentration was chosen because of its intermediate effects on EP cell migration and outgrowth in overnight cultures (described below), and because similar concentrations of Ephrin- and Eph-Fc fusion proteins have produced physiological responses in other culture systems (e.g. St. John et al., 2000; Santiago and Erickson, 2002; Fu et al., 2007). After the application of our Fc constructs, the preparations were allowed to develop for another 4 hr at RT, then fixed and immunostained with anti-TM-MsFas II to visualize the leading growth cones and associated filopodia of the EP cells. To label the midline muscle cells, we also immunostained these preparations with the

monoclonal antibody 4E11; this antibody was originally isolated from a panel of antibodies generated against ENS lysates, and it recognizes an unidentified 112 kDa glycoprotein that is selectively expressed by the mid-dorsal and mid-ventral cells of the midgut (L. Kaler and P.F. Copenhaver, unpublished data). Confocal Z-stack images of the preparations were then analyzed to determine the percentage of EP cell filopodia that had extended onto the inhibitory midline regions versus the normal muscle band pathways. Growth cone areas were calculated by outlining each growth cone (using Adobe Photoshop; San Jose, CA), followed by quantification of the profiles with ImageJ (http://rsb.info.nih.gov/ij/). At least 12 preparations were used for each condition; statistical differences between experimental groups were determined using Student's two-tailed t-test to compare means.

Morpholino experiments.

Morpholino antisense oligonucleotides (morpholinos; Gene Tools, Philomath, OR) were designed against the 5' UTR sequence flanking the initiation codon of the MsEphrin gene (5'-CATAATAAAACTAACACTGCGACAC). Morpholinos (10-50 μM) were diluted in defined saline (supplemented with 10% heat-inactivated normal horse serum, 0.5% penicillin/streptomycin, 0.2% ecdysone, 0.1% insulin and 0.2 M L-glutamine) and transfected into the EP cells of cultured embryos with 0.6% Endo-Porter (Gene Tools; after Summerton, 2005). This range of morpholino concentrations was based on their effectiveness in knocking down endogenous MsEphrin expression in *Manduca* GV-1 cell cultures (unpublished data). Matched sets of embryos were transfected with 3' Carboxyfluorescein (CFSE)-labeled control morpholinos (Gene Tools;

after), or treated with Endo-Porter alone. To optimize the effectiveness of this protocol, embryos were treated with morpholinos starting at 45% of development (±1%) and then allowed to develop for another 48 hr at 28°C. At the end of each experiment, the preparations were double-immunostained with anti-MsEphrin (visualized with Cy3-conjugated secondary antibodies) and anti-MsFas II (visualized with Alexa 488 conjugated secondary antibodies), as described above. To quantify the relative expression levels of both MsEphrin and MsFas II (as an off-target control) in the same EP cells, a 2-μm Z-stack image (consisting of 4 sequential 0.5 μm confocal sections) was taken from the leading groups of neurons in each preparation; a second set of matched optical sections was taken from their fasciculated axons. Each 2-μm stack was then compressed, and fluorescent intensities within regions of interest were quantified using ImageJ software. Background fluorescence levels in each preparation were determined from identical Z-stack images that were collected from adjacent interband muscle regions (areas that were devoid of endogenous MsFas II and MsEphrin expression). The ratios of EP cell-associated fluorescence versus background fluorescence levels were then used to normalize the relative intensities of each fluorochrome in the neurons and their processes; these normalized values were then used to compare relative levels of MsEphrin and MsFas II expression (analyzed independently) between groups. At least 14 preparations were analyzed for each treatment condition, and Students' 2-tailed t-tests were used to compare means for statistical significance. All measurements were performed under linear parameters. Once this analysis was complete, the preparations were re-stained with anti-MsFas II antibodies and the ABC HRP protocol (Vector Laboratories) to quantify

the extent of EP cell migration, axon outgrowth, and number of midline crossover events (as desribed above).

RESULTS

MsEph and MsEphrin are expressed in complementary domains.

As previously described (Copenhaver, 2007; Copenhaver and Taghert, 1989b), the formation of the ENS in *Manduca* requires the precise migration of the enteric neurons (EP cells) from the foregut onto the midgut, prior to their innervation of the visceral musculature (Fig 1). After delaminating from a neurogenic placode in the foregut ectoderm (Copenhaver and Taghert, 1990), the EP cells spread bilaterally around the foregut along the foregut-midgut boundary (from 40-55% of development). By 55% of development, subsets of these neurons (Fig. 1A, red) align with one of eight longitudinal muscle bands (green) that have recently coalesced on the adjacent midgut surface, forming a set of functionally equivalent pathways. Over the next 10 hr (from 55-65% of development; Fig. 1B-C), the neurons migrate rapidly along the muscle bands onto the midgut, followed by a more prolonged period of axon outgrowth along the bands (from 65-80%). Although each EP cell extends an array of exploratory filopodia in advance of its leading process, the neurons and their axons remain closely associated with the muscle band pathways while avoiding the adjacent midline cells (Fig. 1A-C, blue) and lateral interband regions. Only after migration and axon outgrowth is complete (~80% of development) do the neurons extend terminal branches laterally, providing a

diffuse innervation of the midgut musculature (Copenhaver and Taghert, 1989b; Wright et al., 1998).

Previous studies showed that the guidance of the EP cells along the midgut bands is regulated in part by the adhesion receptor *Manduca* Fasciclin II (MsFas II), an immunoglobulin (Ig)-family receptor that is the orthologue of NCAM and OCAM in vertebrates (Grenningloh et al., 1991; Hamlin et al., 2004). MsFas II is simultaneously expressed by both the neurons and the muscle band cells during this period (Fig. 1E, H, K), and manipulations in embryo culture have shown that homophilic interactions between MsFas II on the two cell types promotes migration and outgrowth along these pathways (Wright and Copenhaver, 2000; Wright et al., 1999). In contrast, the expression patterns of MsEph and MsEphrin in the developing ENS suggest that they regulate the behavior of the EP cells at the midline. MsEphrin is expressed only by the migrating neurons and their leading processes (Fig. 1D-L, red), while MsEph is expressed by midline muscle cells on the dorsal and ventral surfaces of the gut (blue cells in Fig. 1D-L; see also (Coate et al., 2007). At higher magnification, MsEphrin-positive filopodia from the EP cells (Fig. 1G-I; arrows) and their growth cones (Fig. 1J-L; arrows) can be seen extending across the surface of the MsFas II-positive muscle bands ("b") and up to the midline cells (which express the MsEph receptor; asterisks), but only rarely do they extend onto these midline regions, suggesting that they stall or turn away from the midline cells upon contacting them (e.g. Fig. 1J-L, arrowhead; (Copenhaver, 2007). Thus, MsEphrin ligands and MsEph receptors are expressed in strictly complementary domains within the developing ENS.

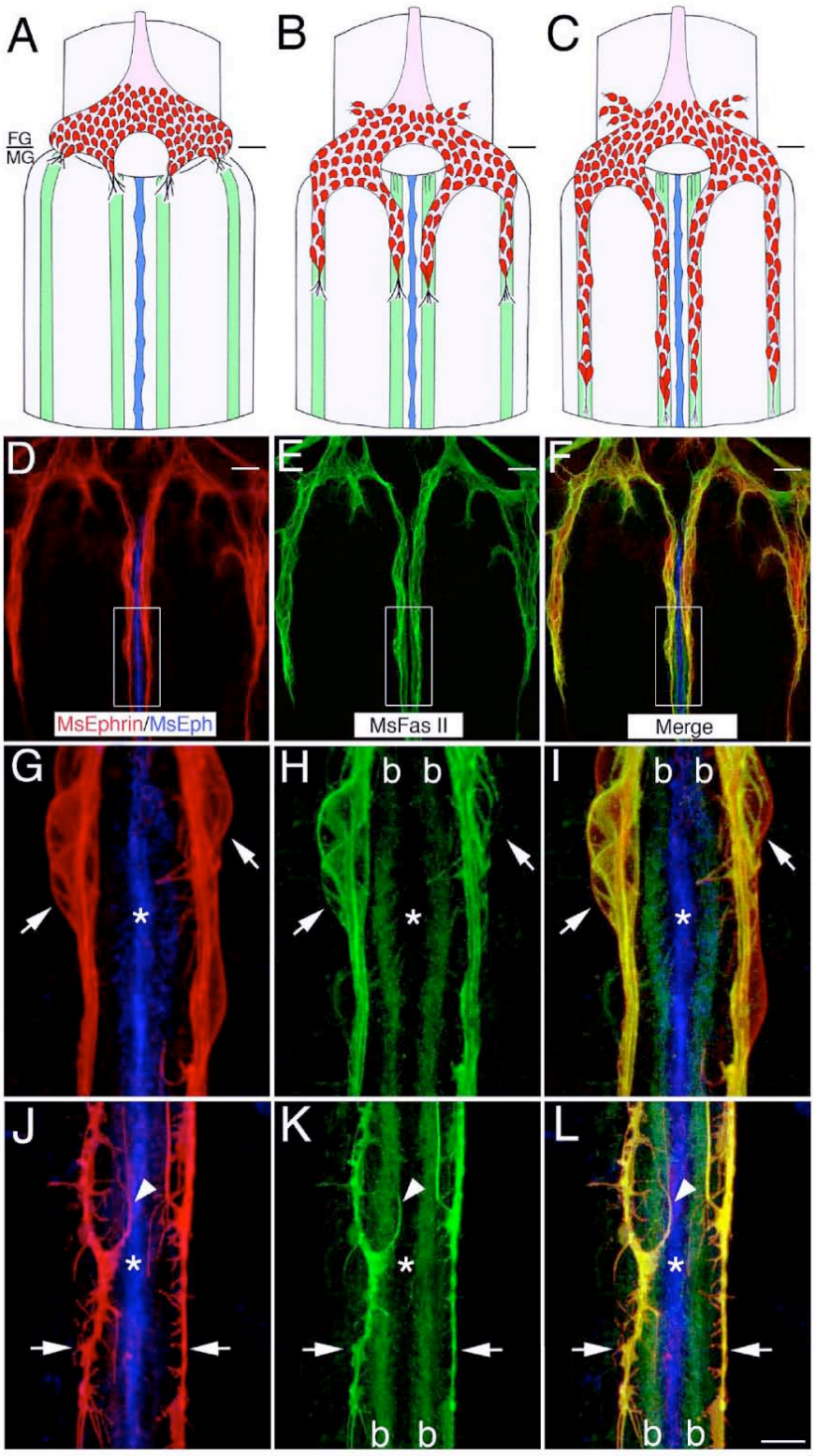


Figure 1.

Figure 1. MsEphrin and MsEph are expressed in complementary patterns within the developing ENS. *A-C*: schematic representation of the migration pattern of neurons that form the enteric plexus ("EP cells"; red). *A*: by ~55% of development, all of the EP cells express MsEphrin, and subsets of the neurons have begun to align with each of eight muscle band pathways (green), which express MsFas II (the EP cells also express MsFas II; not illustrated in *A-C*). Only the four dorsal muscle bands are shown. *B*: at ~60% of development, the EP cells migrate posteriorly along the muscle bands while avoiding the midline muscles (blue), which express MsEph. *C*: at ~65% of development, the EP cells have completed migration, but they continue to extend axonal processes along the band pathways while avoiding the midline. FG/MG = the foregut-midgut boundary. *D*: confocal micrograph of a 65% embryo stained with antibodies against MsEphrin (labeling the neurons; red) and MsEph (labeling the midline; blue). *E*: same preparation counterstained with anti-MsFas II (labeling both the neurons and the muscle bands; green). *F*: merged view of *D* and *E*. *G-I*: enlarged views of the boxed areas in *D-I*. *G*: MsEphrin antibodies (red) label the EP cell somata (arrows) and leading processes, while MsEph antibodies (blue) label the midline cells (asterisk). *H*: MsFas II antibodies (green) label both the neurons (arrows) and muscle bands (b) but not the midline cells (asterisk). *I*: merged view of *G-H*; the EP cells migrate and extend axons along the muscle bands (green) but not across the midline cells (blue). *J-L*: distal processes extended by the EP cells at a more posterior position on the midgut (not visible in *D-I*). *J*: MsEphrin antibodies (red) label the growth cones and filopodia of the EP cells that have grown along the band pathways; MsEphrin-positive filopodia (arrowhead) typically extend up to but not across the midline cells (asterisks), which express MsEph (blue). *K*: MsFas II antibodies label both the EP cell processes and the muscle band pathways (b) but not the midline cells (asterisk). *L*: merged view of *J-K*. Scale bar = 50 μm, in *A-F*; 7 μm in *G-L*

Blocking endogenous MsEphrin-MsEph receptor interactions induces aberrant midline crossing.

Based on the foregoing patterns of expression, we proposed that repulsive interactions mediated by MsEphrin on the EP cells and MsEph receptors on the midline cells prevent the neurons from crossing the gut midline. To test this hypothesis, we generated affinity-purified MsEphrin-Fc fusion proteins designed to target endogenous MsEph receptors within the ENS. When analyzed in Western blots run under reducing conditions, MsEphrin-Fc behaved as a monomer with an apparent mass of ~55 kDa (Fig.

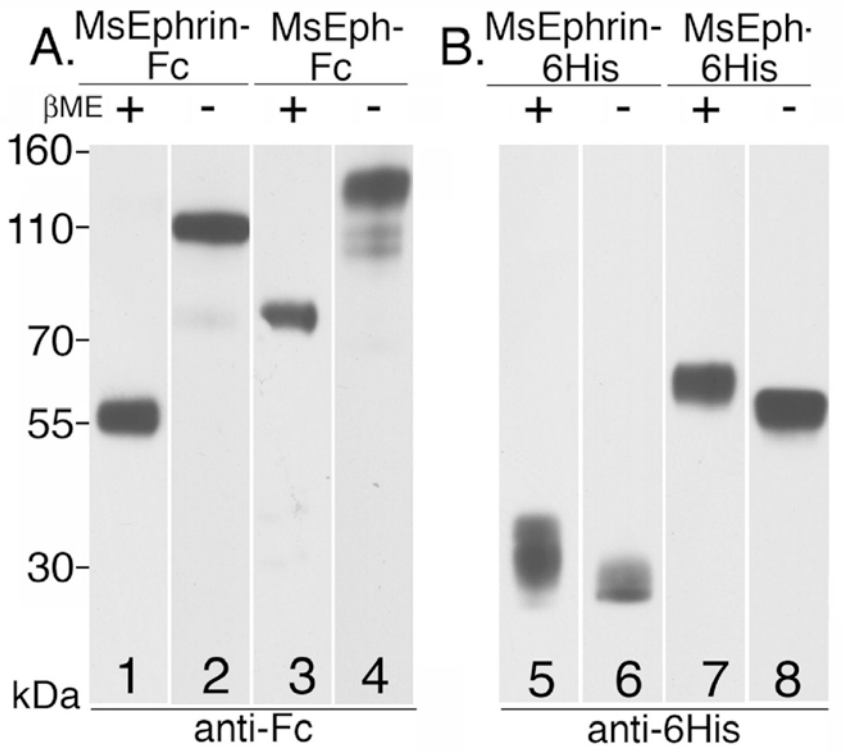


Figure 2. Immunoblot analysis of Fc- and 6His-tagged fusion proteins. Lanes 1-2: 20 ng MsEphrin-Fc was electrophoresed under reducing conditions (+βME; lane 1) or non-reducing conditions (-βME; lane 2) and detected with anti-Fc antibodies. Under reducing conditions, MsEphrin-Fc migrated with the apparent molecular size of ~55 kDa, equivalent to the predicted size of the monomeric protein, while under non-reducing conditions, the protein migrated primarily as a dimer (~110 kDa). Lanes 3-4: 20 ng MsEph-Fc was electrophoresed under reducing conditions (+βME; lane 3) or non-reducing conditions (-βME; lane 4) and detected with anti-Fc antibodies. Under reducing conditions, MsEph-Fc migrated with the apparent molecular size of ~86 kDa, equivalent to the predicted size of the monomeric protein, while under non-reducing conditions, the protein migrated primarily as a dimer (~150 kDa). Lanes 5-6: 20 ng of MsEphrin-6His was electrophoresed under reducing conditions (+βME; lane 5) or non-reducing conditions (-βME; lane 6) and detected with anti-6His antibodies. MsEphrin-6His migrated primarily as a monomer under both conditions (predicted size = 28 kDa). Lanes 7-8: 20 ng of MsEph-6His was electrophoresed under reducing conditions (+βME; lane 7) or non-reducing conditions (-βME; lane 8) and detected with anti-6His antibodies. MsEph-6His migrated primarily as a monomer under both conditions (predicted size = 62 kDa); the slightly smaller apparent size seen under non-reducing conditions may reflect incomplete denaturation of the protein.

2, lane 1), as predicted from its primary amino acid sequence. In contrast, under non-reducing conditions, the protein migrated with the apparent size of a dimer (~110 kDa; Fig. 2, lane 2). Likewise, when we examined the molecular mass of MsEphrin-Fc in physiological saline by analytical ultracentrifugation, the protein was detected primarily as a dimer (~130 kDa; data not shown). These results are consistent with current models of Ephrins and Eph receptors in other systems, which predict that both the ligands and receptors initially form dimers and then interact with a 2:2 stoichiometry (as illustrated in Fig. 3A; see Chrencik et al., 2006; (Himanen et al., 2001; Pabbisetty et al., 2007).

Previously, we showed that Fc fusion proteins can be used to detect bioavailable pools of MsEphrin and MsEph receptors within the developing ENS (Coate et al., 2007). When we applied MsEphrin-Fc to the ENS of cultured embryos, we found that it selectively bound the midline muscle cells of the midgut (Fig. 3B-C), a pattern that precisely corresponded with the endogenous expression of MsEph receptors by these cells (Fig. 1). We then tested whether applying MsEphrin-Fc to the ENS at the onset of EP cell migration (targeting MsEph receptors at the midline) affected the subsequent behavior of the neurons and their processes. As shown in figure 3, this treatment caused a substantial number of EP cells to migrate and extend neurites abnormally across the midline (Fig. 3E & F; asterisks indicate the mid-dorsal midline cells in each panel). Similar pathfinding errors were seen in their axonal trajectories, resulting in the deviation of both fasciculated axon bundles (Fig 3H) and what appeared to be individual branches (Fig. 3I) across the midline (c.f. Copenhaver and Taghert, 1989a; 1989b). In contrast, embryos treated with normal medium or with control Fc proteins did not exhibit these types of crossover errors by either the EP cells (Fig. 3D) or their axons (Fig. 3G), which

remained confined to their normal band pathways. Quantification of the number of crossover events per embryo in these experiments (Fig. 4A) showed that control Fc proteins caused no significant effects at concentrations as high as 100 μg/ml: as in untreated control embryos (histogram 'C'), these preparations exhibited only rare instances of neurites that strayed across the midline. In contrast, treatment with MsEphrin-Fc (from 0.1-50 μg/ml) caused a concentration-dependent increase in crossover events: at 0.1 μg/ml, MsEphrin-Fc induced more than twice the average number of crossover events (compared to control Fc proteins), while treatments with 50 μg/ml induced a maximal 6-fold increase in number of cells and processes that deviated across the midline. Similar concentrations of Ephrin- and Eph-Fc fusion proteins have been shown to induce physiological responses in a variety of other culture assays (e.g. Santiago and Erickson, 2002; Kasemeier-Kulesa et al., 2006; Vidovic et al., 2006; Fu et al., 2007), while higher concentrations did not produce significantly more crossover events in the developing ENS (data not shown). Despite these defects, MsEphrin-Fc treatments did not significantly affect the overall extent of EP cell migration or axonal outgrowth along the normal muscle band pathways (Fig. 4B-C), nor did they cause any other obvious abnormalities in ENS development. These results suggest that interactions between MsEphrin on the EP cells and MsEph receptors on the midline muscles of the gut normally prevent inappropriate midline crossing by the neurons, but they do not apparently play a predominant role in regulating other aspects of neuronal migration and outgrowth.

Manipulations designed to pre-cluster Ephrin-Fc and Eph-Fc fusion proteins have often been found to potentiate the activation of their cognate binding partners in cell

culture (Davis et al., 1994; Hattori et al., 2000; Palmer and Klein, 2003). However, pre-clustering MsEphrin-Fc with anti-Fc antibodies caused only a slight increase in the frequency of crossover events that was not significantly different from the effects of unclustered proteins at the same concentrations (Fig. 4A; compare $\pm$ IgG). Given our evidence that MsEphrin-Fc spontaneously forms dimers but not multimers under physiological conditions (Fig. 2; and data not shown), these results indicate that higher order complexes of MsEphrin were not required for the effects seen in this *in vivo* context.

Previous studies have indicated that Ephrin-Fc dimers may either block endogenous ligand-receptor interactions (thereby preventing signal transduction) or activate Eph receptors (inducing 'forward' signaling; Krull et al., 1997; (Contractor et al., 2002; Stein et al., 1998). To determine whether MsEphrin-Fc treatments induced midline crossing in the ENS by blocking endogenous MsEphrin-MsEph interactions or by over-stimulating MsEph receptor activation, we generated monomeric MsEphrin-6His fusion proteins that, based on current models, should bind but not activate MsEph receptors (cf. Davis et al., 1994). As shown in Fig. 2 (lanes 5-6), immunoblots of purified MsEphrin-6His showed that it behaved as a monomer under both reducing and non-reducing conditions (predicted size = 28 kDa), in contrast to the spontaneous dimers formed by MsEphrin-Fc (Fig. 2, lane 2). Notably, treating the developing ENS with monomeric MsEphrin-6His induced abnormal midline crossing by both the migrating EP cells (Fig. 3K) and their growing processes (Fig. 3L), at frequencies that were comparable to the effects of dimeric MsEphrin-Fc (Fig. 4A). As with MsEphrin-Fc treatments, however, MsEphrin-6His did not induce significant alterations in the overall distance of migration

or axonal outgrowth along the normal band pathways (Fig. 4B, C), suggesting that endogenous MsEphrin-MsEph interactions specifically regulate the behavior of the migratory neurons at the midline.

The effects of these manipulations were also apparent in *camera lucida* images taken from the different culture preparations. In mock-treated preparations or preparations treated with control Fc proteins (Suppl. Fig. 2, panel B), both the neurons and their axons remained closely apposed to the normal muscle band pathways throughout the course of migration and outgrowth. (Panel A illustrates the position of the pre-migratory EP cells at the onset of these experiments). In contrast, embryos treated with either MsEphrin-Fc (Suppl. Fig. 2, panel C) or MsEphrin-6His (not shown) exhibited a substantial number of aberrant crossover events at the midline, although the overall extent of EP cell migration and outgrowth was unaffected. Since both monomeric MsEphrin-6His and dimeric MsEphrin-Fc induced identical crossover phenotypes without significantly altering neuronal motility, these results argue that their effects were primarily due to interference with endogenous MsEphrin-MsEph interactions, rather than the induction of a forward signaling response through MsEph receptors on the midline cells.

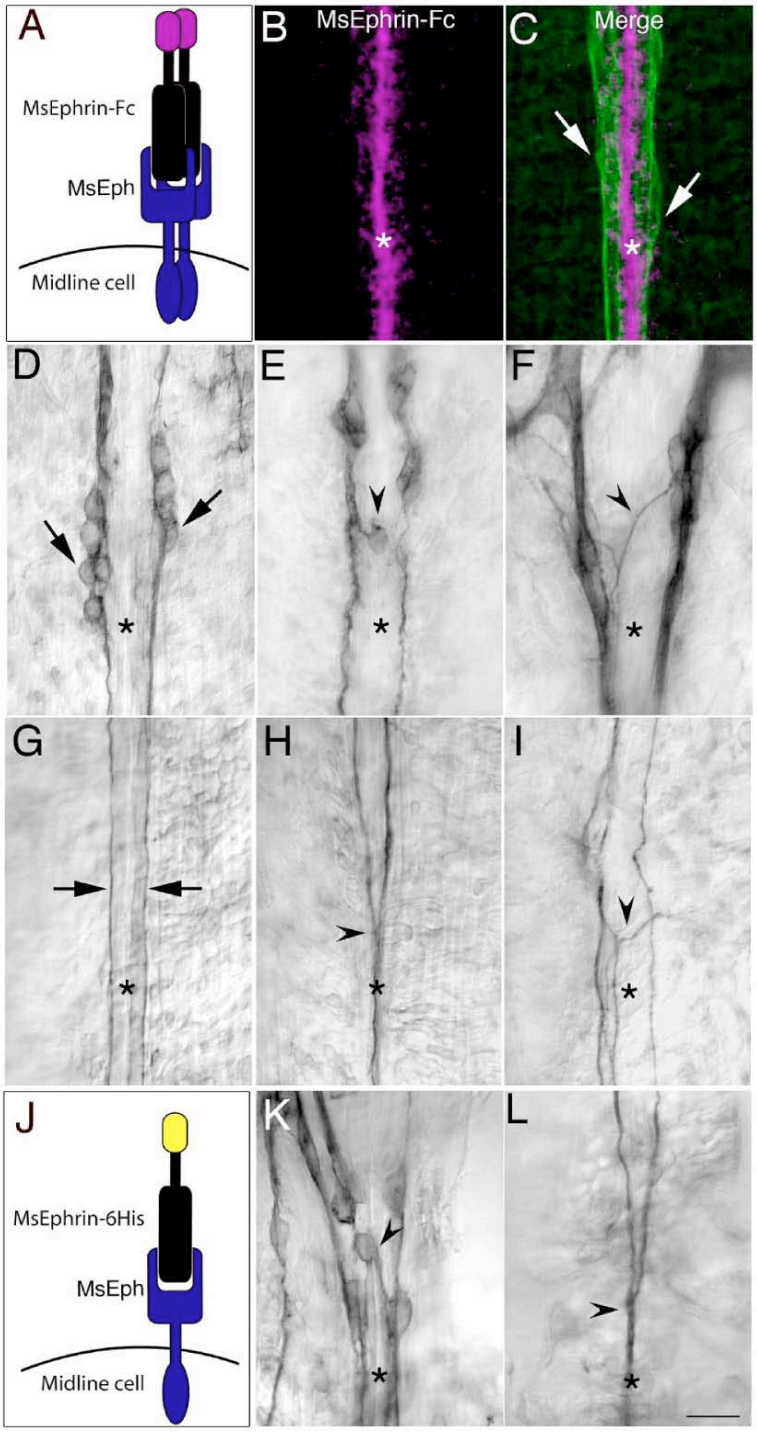
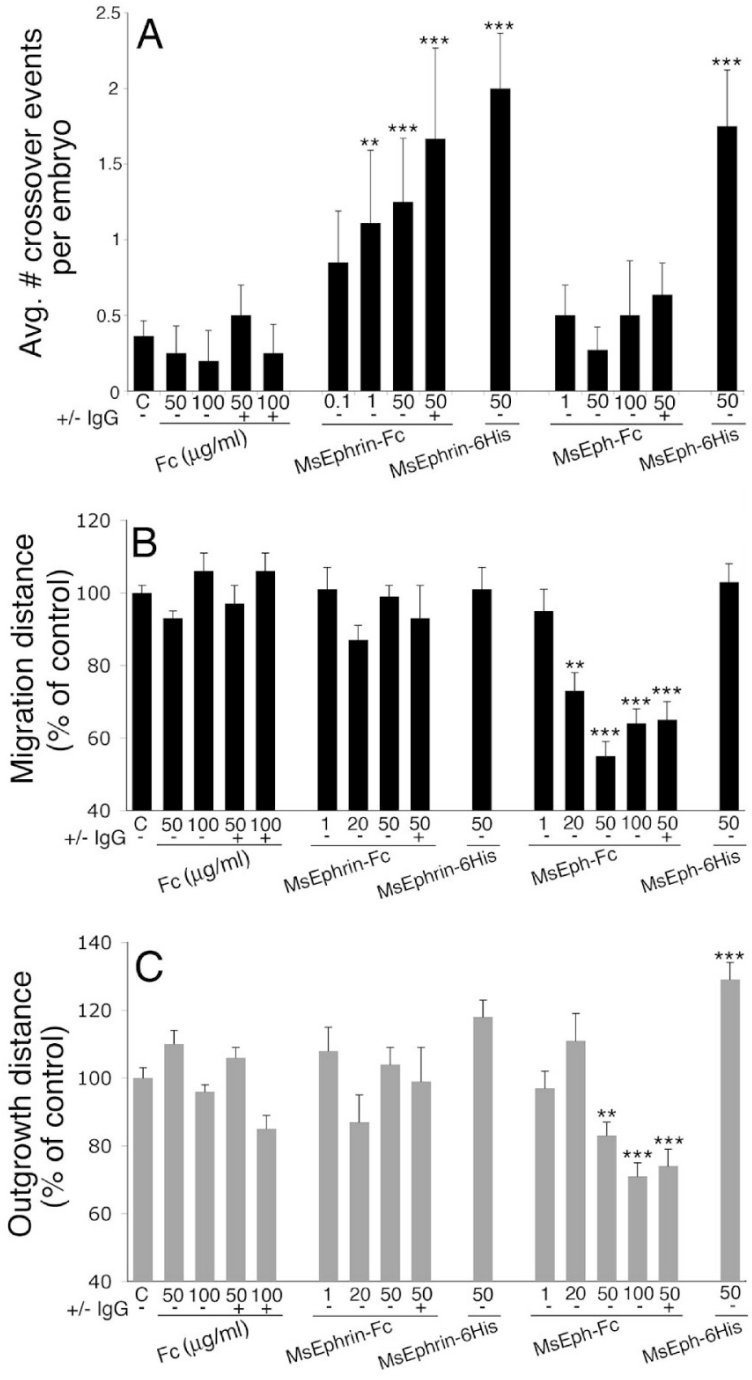

A
MsEphrin-Fc
MsEph
Midline cell
B
MsEphrin-Fc
C
Merge
D
E
F
G
H
I
J
MsEphrin-6His
MsEph
Midline cell
K
L

Figure 3.

Figure 3. Treating the developing ENS with MsEphrin fusion constructs induced midline crossing by the EP cells and their processes. *A*: schematic diagram illustrating the interaction between dimeric MsEphrin-Fc fusion proteins (black, with magenta Fc tag) and MsEph receptors on the midline cells (blue). *B*: MsEphrin-Fc selectively binds the midline cells of the midgut (magenta; asterisk) when applied to the ENS of unfixed tissue (at 4^{o}C; detected with anti-Fc antibodies). *C*: counterstaining the same preparation with anti-MsFas II antibodies (green) shows that MsEphrin-Fc does not label the EP cells (arrows) or the muscle band pathways. *D-I*: embryos that were treated with Fc proteins for 24 hr in culture, then immunostained with anti-MsFas II to reveal the EP cells and their processes. *D & G:* embryo that was treated with control Fc protein; the EP cells (*D*, arrows) and their processes (*G*, arrows) traveled normally along their muscle band pathways without crossing onto the midline cells (asterisk). *E, F, H, & I*: embryos that were treated with MsEphrin-Fc showed a variety of midline crossover phenotypes. *E:* example of an EP cell that migrated ectopically across the midline. *F*: example of an EP cell neurite that grew across the midline. *H*: preparation in which the distal axon fascicles of the EP cells had merged on the midline cells. *I*: preparation in which the distal process of an EP cell defasciculated and crossed the midline. *J*: schematic diagram illustrating the interaction between monomeric MsEphrin-6His fusion proteins (black, with yellow 6His tag) and MsEph receptors on the midline cells (blue). *K-L:* embryos that were treated with MsEphrin-6His showed similar examples of midline crossing by the EP cells (*K*) and distal axons (*L*). Arrowheads in all panels indicate crossover events; asterisks indicate the midline. Scale bar = 40 μm for all of the micrographs.

Figure 4. Quantitative analysis of the effects MsEphrin and MsEph fusion proteins on the behavior of the EP cells and their processes. *A*: histogram depicting the average number of crossover events per embryo (including cells, axon fascicles, and isolated neurites). Whereas crossover events were only rarely seen in untreated control preparations (C) or preparations treated with control Fc protein, treatment with dimeric MsEphrin-Fc induced a concentration-dependent increase in all types of crossover events. Monomeric MsEphrin-6His and monomeric MsEph-6His proteins both induced crossover events at the same frequency as dimeric MsEphrin-Fc, while pre-clustering the Fc fusion proteins with anti-Fc antibodies ($\pm$IgG) did not induce significantly more crossover events than the unclustered proteins. In contrast, dimeric MsEph-Fc did not induce crossovers more frequently than in controls. *B* and *C*: histograms illustrating the extent of EP cell migration and outgrowth along the muscle band pathways in each treatment group (normalized to matched control preparations). Treatments with either dimeric MsEphrin-Fc or monomeric MsEphrin-6His caused no significant alterations in the distance traveled by the neurons and their processes compared to controls; treatment with monomeric MsEph-6His also did not affect migration but caused significant enhancement of axon outgrowth. In contrast, treatment with dimeric MsEph-Fc caused a concentration-dependent inhibition of both migration and outgrowth along the band pathways. The effects of pre-clustered MsEph-Fc ("+IgG") were not significantly different than unclustered dimeric proteins at the same concentrations. Fc protein concentrations are given in μg/ml. "+/- IgG" indicates whether the Fc proteins were pre-clustered with an anti-Fc IgG before their application. Statistical analysis was performed using Student's t-test; **$p<0.01$. ***$p<0.001$.


A
Avg. # crossover events per embryo
2.5
2
1.5
1
0.5
0
**
***
***
***
***
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+/- IgG - - - + + - - - + - - - - + -
Fc (µg/ml)
MsEphrin-Fc
MsEphrin-6His
MsEph-Fc
MsEph-6His
B
Migration distance (% of control)
120
100
80
60
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Fc (µg/ml)
MsEphrin-Fc
MsEphrin-6His
MsEph-Fc
MsEph-6His

Figure 4.

Activating MsEphrin ligands on the EP cells inhibits migration and axonal outgrowth.

To target endogenous MsEphrin ligands expressed by the migratory EP cells, we also generated Fc-tagged fusion constructs containing the extracellular domain of the MsEph receptor (Coate et al., 2007). As with MsEphrin-Fc, Western blots of MsEph-Fc proteins showed that it migrated as a monomer in reducing conditions, with the predicted molecular size of 86 kDa (Fig. 2, lane 3). However, when run in non-reducing conditions, it migrated as a dimer (~150 kDa; lane 4). When examined in solution by analytical ultracentrifugation, MsEph-Fc proteins also behaved primarily as dimers (~174 kDa), although a small percentage of the total protein migrated as larger aggregates (not shown).

Using MsEph-Fc proteins to label cultured embryos (at 4^{o}C), we found that they specifically bound to the EP cells and their processes but not the underlying muscle bands or the midline cells (Fig. 5A-C); this pattern corresponds precisely with the neuronal-specific expression of MsEphrin within the developing ENS (see also Coate et al., 2007). However, when we exposed the EP cells to dimeric MsEph-Fc at the onset of migration, we observed no significant increase in the frequency of midline crossing events, in marked contrast to the effects of either MsEphrin-Fc and MsEphrin-6His proteins (Fig. 4A). Instead, MsEph-Fc treatments caused a dramatic inhibition in the extent of migration and outgrowth by the neurons, without inducing aberrant sprouting onto the midline (Fig. 5E-F). EP cells in these preparations often stalled soon after leaving the foregut-midgut boundary (Fig. 5E; indicated by black bars) or, at higher concentrations, completely failed to migrate (Fig. 5F; see also Suppl. Fig. 2, panels D-E). The inhibitory

effects of MsEph-Fc were both concentration-dependent (over a range of 1-50 μg/ml) and statistically significant (Fig. 4B, C), in marked contrast to the minor changes in the overall distance of migration and outgrowth caused by either MsEphrin-Fc or MsEphrin-6His. Pre-clustering MsEph-Fc with anti-Fc antibodies did not further enhance its potency in inhibiting neuronal migration and outgrowth (Fig. 4B-C; "±IgG"). Thus, as with MsEphrin-Fc, the formation of higher order complexes did not appear necessary for the biological activity of MsEph-Fc in this *in vivo* context.

The inhibitory effect of MsEph-Fc proteins on EP cell motility might be due to a paradoxical response caused by blocking endogenous MsEphrin-MsEph interactions, although our foregoing experiments would predict that such an effect would only cause midline crossing. Alternatively, treatment with MsEph-Fc might overstimulate a reverse signaling event through MsEphrin on the neurons. To discriminate between these possibilities, we designed an MsEph-6His fusion protein that should not form spontaneous dimers or activate reverse signaling through MsEphrin (cf. Davis et al., 1994). As shown in Fig. 2 (lanes 7-8), MsEph-6His did indeed behave as a monomer in both reducing and non-reducing conditions, migrating near its predicted molecular size of 62 kDa. The slightly smaller size of ~57 kDa seen in non-reducing conditions was presumably due to incomplete denaturation of the protein.

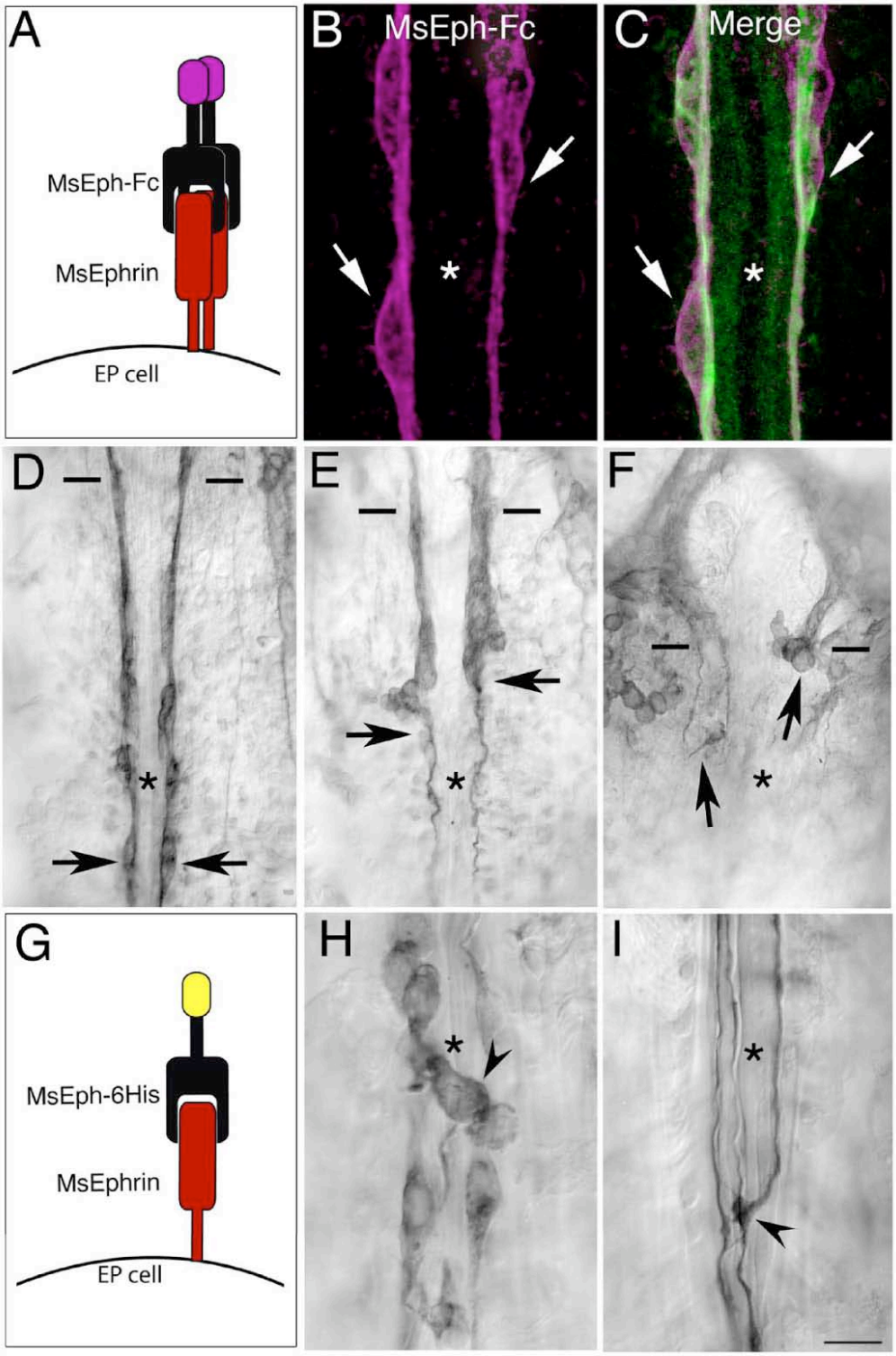

A
MsEph-Fc
MsEphrin
EP cell
B
MsEph-Fc
C
Merge
D
E
F
G
MsEph-6His
MsEphrin
EP cell
H
I

Figure 5.

Figure 5. Dimeric MsEph-Fc proteins inhibited EP cell migration and outgrowth, while monomeric MsEph-6His caused aberrant midline crossovers. *A*: schematic diagram of the interaction between dimeric MsEph-Fc fusion proteins (black, with magenta Fc tag) and MsEphrin ligands on the EP cells (red). *B*: MsEph-Fc selectively binds to the EP cells (magenta; arrows) but not the midline cells (asterisk) when applied to the ENS of unfixed preparation (at 4^{o}C; detected with anti-Fc antibodies). *C*: Counterstaining the same preparation with anti-MsFas II antibodies (green) shows that MsEph-Fc labels the EP cells (arrows) but not the muscle band pathways. *D-F*: embryos that were treated with Fc proteins for 24 hr in culture, then immunostained with anti-MsFas II to reveal the EP cells and their processes. *D*: embryo that was treated with control Fc protein; the EP cells (arrows) and their processes traveled normal distances along their muscle band pathways without crossing onto the midline cells (asterisk). *E*: embryo treated with 20 μg/ml MsEph-Fc showed reduced EP cell migration and outgrowth. *F*: embryo treated with 50 μg/ml MsEph-Fc showed a more severe inhibiton of migration and outgrowth. *G*: schematic diagram of the interaction between monomeric MsEph-6His fusion proteins (black, with yellow 6His tag) and MsEphrin ligands on the EP cells (red). *H-I*: embryos that were treated with MsEph-6His showed ectopic midline crossing by the migratory EP cells (*H*, arrowhead) and their processes (*I*, arrowhead) that were similar to crossover phenotypes caused by MsEphrin-Fc and MsEphrin-6His. Horizontal black bars in *D-F* indicate the foregut-midgut boundary; asterisks in all preparations indicate the midline. Scale bar = 20 μm in *B-C* and *G-I*, 50 μm in *D-F*.

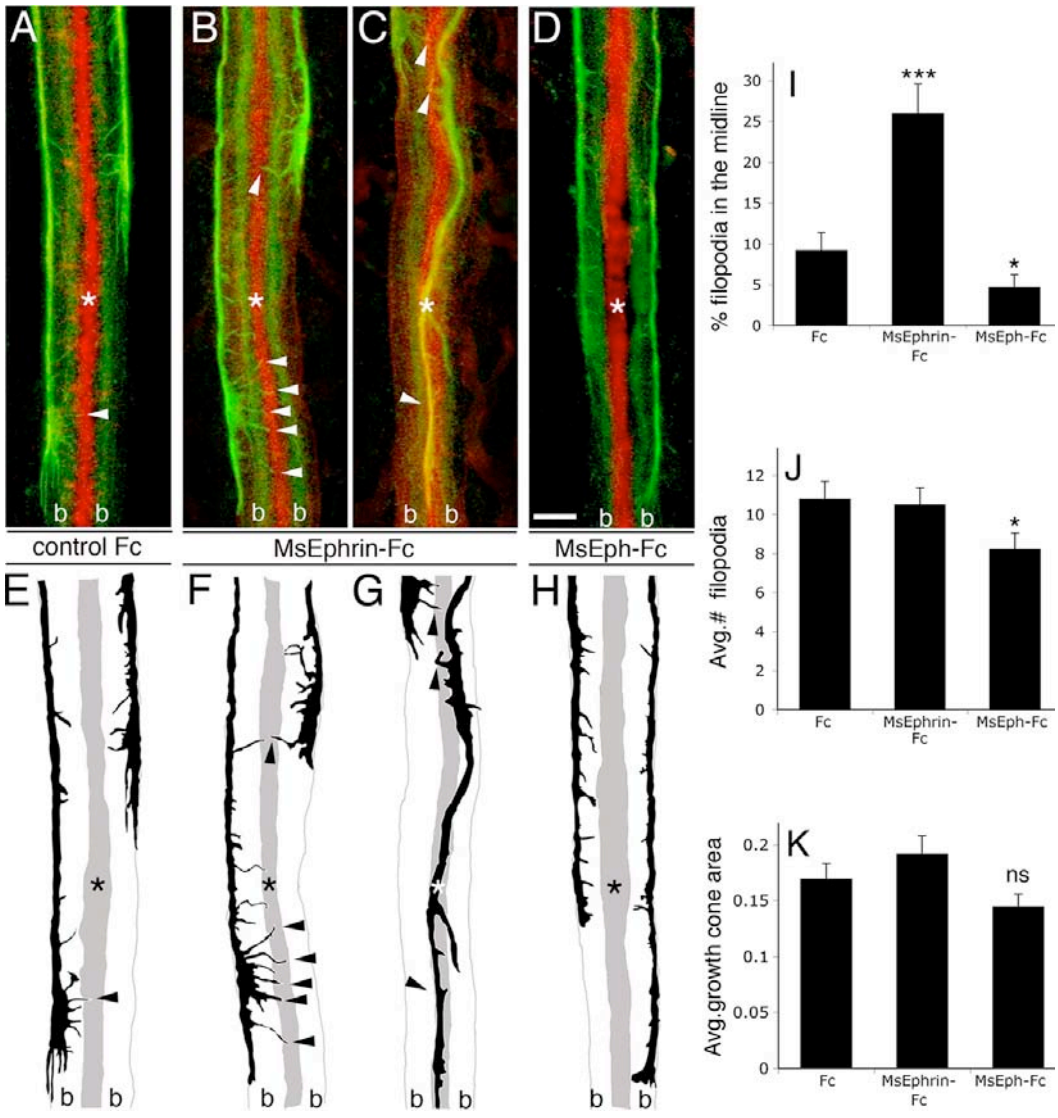
When we treated the migratory EP cells with MsEph-6His, we observed numerous midline crossing events by both the neurons (Fig. 5H) and their processes (Fig. 5I), at frequencies that were comparable to the effects of both MsEphrin-Fc and MsEphrin-6His (Fig. 4A). However, monomeric MsEph-6His proteins did not affect the overall extent of EP cell migration (Fig. 4B), in marked contrast to the inhibitory effects of dimeric MsEph-Fc proteins. These results are consistent with the model that monomeric MsEph-6His proteins acted by blocking endogenous MsEphrin-MsEph receptor interactions, rather than stimulating MsEphrin-dependent signaling. Unexpectedly, MsEph-6His also caused an *increase* in axonal outgrowth along the band pathways (Fig. 4C). However, this effect only became apparent over the protracted period of axonal elongation (from 65-80% of development), possibly reflecting a low, chronic level of endogenous MsEphrin activation in the EP cells that normally modulates

their overall motility. The fact that MsEph-6His monomers induced midline crossing events while MsEph-Fc dimers inhibited migration and outgrowth supports the hypothesis that endogenous MsEph receptors at the midline normally prevent the neurons from crossing these regions via reverse activation of their MsEphrin ligands on the EP cells.

Midline MsEph receptors guide exploratory filopodia on motile neurons.

During the normal migration of the EP cells along the muscle bands, new filopodia from their leading processes continually extend onto the adjacent surfaces of the midgut. Filopodia that remain in contact with the bands (which express MsFas II) tend to become stabilized, thereby promoting growth along these pathways, while filopodia that extend onto adjacent interband regions have comparatively short lifetimes and are typically retracted (Coate et al., 2007; Copenhaver, 2007). Does the presence of bioavailable MsEph receptors on the midline cells prevent MsEphrin-positive filopodia from entering these regions? To investigate this issue, we conducted a series of short-duration experiments using animals between 58-60% of development, a stage when the EP cells and their leading processes advance rapidly onto the midgut ((Copenhaver et al., 1996; Copenhaver and Taghert, 1989b). After treating these preparations with the different Fc fusion proteins for 3 hr in culture (at 28^{o}C), we double-immunostained them with anti-TM-MsFas II (to visualize the neuronal processes and underlying muscle bands) and 4E11 (to label the midline interband cells). High-resolution confocal microscopy was then used to analyze the distributions of filopodia associated with the leading processes of the EP cells.

In preparations treated with control Fc protein, only 9% of the filopodia were found in contact with the midline cells (Fig. 6A, E, arrowheads; Fig. 6I). In contrast, treatments with MsEphrin-Fc (targeting MsEph receptors on the midline cells) led to a significant increase (26%) in the number of filopodia that had extended onto the midline cells and failed to retract (Fig 6B-C & F-G, arrowheads; Fig. 6I), although there were no significant changes in the total number of filopodia (Fig. 6J) or average growth cone area (Fig. 6K) associated with the leading processes. Figure 6 also illustrates the range of phenotypes that were produced by treating the ENS with MsEphrin-Fc: in some preparations, an increased number of filopodia had entered the midline without affecting the orientation of the growth cone (Fig. 6B, F), while in more extreme cases, the entire growth cone had migrated off its normal muscle band pathway and onto the midline region (Fig. 6C, G). The opposite effect was produced by treatments with MsEph-Fc: the leading processes of the EP cells in these preparations had a more tapered appearance (Fig. 6D, H) and extended significantly fewer filopodia onto the midline, compared to controls (Fig. 6I). We also detected a small but significant reduction in the total number of filopodia per leading process (Fig. 6J), although the overall size of their growth cones was not significantly reduced (Fig. 6K). Possibly, this relatively subtle effect reflected the presence of other guidance cues in the ENS that help stabilize the leading processes, thereby preventing catastrophic growth cone collapse. Nevertheless, these results suggest that during normal ENS development, reverse signaling via MsEphrin restricts the local filopodial dynamics of the leading processes extended by the migrating EP cells, thereby preventing the neurons from growing onto the midline regions of the gut.


A
B
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MsEph-Fc
J
Avg.# filopodia
K
Avg.growth cone area
ns

Figure 6.

Figure 6. The effects of short-term treatments with MsEphrin-Fc and MsEph-Fc fusion proteins on the growth cones of the EP cells. Cultured embryos were treated for 3 hr at RT with either 20 μg/ml control Fc, MsEphrin-Fc, or MsEph-Fc, then fixed and stained for MsFas II (green; to label the EP cell growth cones and underlying muscle bands) and 4E11 (red; to label the midline cells). *A-D* are representative micrographs of the different treatments; *E-F* are corresponding silhouettes from *A-D* used for the quantification shown in *I-K*. Asterisks and grey stripes in Figures *A-H* indicate the position of the midline cells. *A* & *E:* preparation treated with control Fc showed a small number of filopodia (e.g. arrowhead) that had contacted the midline (asterisk). *B & F:* example of preparation treated with MsEphrin-Fc (targeting the midline MsEph receptors) that showed a marked increase in the number of filopodia entering the midline (arrowheads); *C & G*: another preparation treated with MsEphrin-Fc in which an entire growth cone entered the midline. *D* & *H*: embryo treated with MsEph-Fc (targeting MsEphrin on the EP cell growth cones) showed no detectable filopodia in contact with the midline; the total number of filopodia was also slightly reduced. *I*: Percent of filopodia per growth cone that had entered the midline in the different treatment groups; MsEphrin-Fc treatment resulted in significantly more filopodia that contacted the midline compared to controls, while MsEph-Fc treatment led to significantly fewer midline filopodia. *J*: average total number of filopodia associated with the EP cell growth cones in the different treatment groups (counted within 50 μm of the most distal growth cone tip). MsEph-Fc treatment resulted in significantly fewer filopodial processes per growth cone compared to controls, while MsEphrin-Fc treatment had no significant effect. *K*: average growth cone area (in arbitrary units) calculated from silhouette profiles (see *E-H*). MsEph-Fc treatments caused a minor reduction in growth cone size, but this effect was not significant (NS). Scale bar = 5 μm for *A-H*. Samples sizes for the different conditions were as follows: for Fc treatments, n = 12; for MsEphrin-Fc, n = 20; for MsEph-Fc, n = 18. Significant differences in *I-K* were calculated with Student's 2-tailed t-test; *$p<0.05$, ***$p<0.001$.

Knock-down of MsEphrin expression in the EP cells with morpholinos leads to midline crossing.

If MsEphrin-MsEph interactions are indeed required for regulating the behavior of the EP cells at the midline, then inhibiting the expression of either protein should also lead to aberrant midline crossing by the neurons and their processes. To address this issue, we treated the ENS of cultured embryos with morpholinos specifically targeting the 5' UTR of the MsEphrin gene, using Endo-Porter (0.6%) as a delivery reagent; at this concentration, Endo-Porter had no deleterious effects on embryonic development (data

not shown). When we introduced CFSE-labeled control morpholinos (1-50 μM) into the developing ENS, we detected the accumulation of the morpholinos in virtually all of the EP cells (Suppl. Fig. 3), as well as in adjacent muscle cells and other non-neural cells (as expected with this delivery system). However, introduction of these and other control morpholinos had no appreciable effects on the expression or localization of either MsEphrin or MsFas II in the neurons, nor did they affect EP cell migration and outgrowth. As shown in figure 7, labeling these control preparations at the end of the culture period with anti-MsEphrin antibodies revealed normal patterns of MsEphrin expression on both the somata and axons of the EP cells (Fig. 7A, G; compare with Fig. 1). Likewise, control morpholinos at concentrations up to 50 μM had no detectable effect on MsFas II expression (Fig. 7B, H), providing further evidence that our treatment conditions did not produce any obvious non-specific effects on gene expression or migratory behavior by the EP cells.

In contrast, treating the developing ENS with 50 μM MsEphrin-specific morpholinos caused a dramatic reduction in the level of MsEphrin expression in the EP cell somata and processes (Fig. 7D, J). As with the control morpholinos, MsEphrin-specific morpholinos had no detectable effect on MsFas II expression levels in these neurons (Fig. 7E, K), indicating that they did not produce generalized off-target effects on gene expression. However, the MsEphrin morpholinos did induce a dramatic increase in midline crossovers (as revealed by MsFas II immunostaining; Fig. 7E-F, K-L), an effect that was not seen in preparations treated with control morpholinos (Fig. 7B-C, H-I). Notably, those neurons and processes that grew inappropriately across the midline expressed little or no MsEphrin (Fig. 7D-L, arrowheads).

To quantify the effects of these treatments on MsEphrin expression by the EP cells, we estimated the relative levels of MsEphrin-specific immunofluorescence in each preparation (detected with a Cy3-conjugated secondary antibody) by normalizing against background fluorescent levels in adjacent, MsEphrin-negative regions of the gut. As an internal control for potential non-specific effects of the morpholinos, we simultaneously quantified the relative levels of MsFas II-specific immunofluorescence in these same regions (detected with an Alexa 488-conjugated secondary antibody), again by normalizing against background levels within each preparation. Supplemental figures 4 and 5 illustrate the methods used to determine the relative levels of MsEphrin and MsFas II immunofluorescence that were associated with the migratory neurons and their growing processes. We then used these normalized values to compare the relative effects of the morpholino treatments on MsEphrin and MsFas II expression independently.

As shown in figure 7M, when we compared preparations treated with control morpholinos (black histograms) versus embryos treated with MsEphrin-specific morpholinos (gray histograms), no significant differences were detected in the relative intensity of MsFas II-associated immunofluorescence in either the somata or axons of the EP cells. (The enhanced levels of MsFas II in the axons compared to the somata reflects the developmental trafficking of this adhesion receptor to regions of active motility; Wright et al., 2000). In contrast, the relative intensity of MsEphrin-associated immunofluorescence was dramatically reduced in preparations treated with MsEphrin morpholinos, compared with preparations treated with control morpholinos (Fig. 7N). Relative levels of MsEphrin immunofluorescence were reduced by more than 90% in the somata of the EP cells and by 45% in their axons.

Concurrent with their selective effect on MsEphrin expression, treatments with MsEphrin morpholinos (1-50 μM) caused a concentration-dependent increase in midline crossing events (Fig. 7O), whereas preparations treated with control morpholinos exhibited only rare crossovers by scattered neurites, as seen in earlier controls (compare with Fig. 4A). Thus, knocking down MsEphrin expression produced the same crossover phenotypes caused by blocking endogenous MsEphrin-MsEph interactions in the ENS. We also measured the effects of the morpholinos on the average total distance traveled by the EP cells along their normal muscle band pathways. As illustrated in Figure 7P, neither the control nor MsEphrin-specific morpholinos reduced the overall extent of migration and outgrowth (Fig. 4B-C; Fig. 6); in fact, MsEphrin morpholinos induced a slight *enhancement* of migration along the band pathways, increasing average migration distances by up to 25%, compared to controls. This result is similar to the increase in axonal outgrowth caused by treatments with MsEph-6His (Fig. 4C), again suggesting that MsEphrin-MsEph receptor interactions may modulate the overall motility of the EP cells in conjunction with preventing midline crossing. Why knocking down MsEphrin expression preferentially affected migration versus outgrowth is unclear, but may be related to our observation that the morpholino treatments depleted MsEphrin levels in the neuronal somata more efficiently than in their leading axons, where existing protein may be preferentially retained (Fig. 7D & J and Suppl. Figs. 4-5). The loss of MsEphrin may therefore delay the normal transition from migration to outgrowth indirectly, possibly by modulating the local cytoskeletal dynamics that distinguish these behaviors (Copenhaver, 2007). Nevertheless, these effects are in marked contrast to the strong inhibitory effects of dimeric MsEph-Fc on both migration and outgrowth,

In summary, these experiments provide evidence that MsEphrin-MsEph receptor interactions normally prevent the migratory EP cells and their processes from inappropriately crossing the midline of the developing ENS. They also support the model that reverse signaling through this GPI-linked Ephrin regulates neuronal guidance within the developing ENS, an effect that we propose is mediated by the local activation of this response within the leading processes of the migrating neurons (summarized in figure 8). As discussed below, the integration of this reverse signaling response with input from other attractive and repulsive cues encountered on the midgut pathways ultimately determines the final distribution of neurons in the mature ENS.

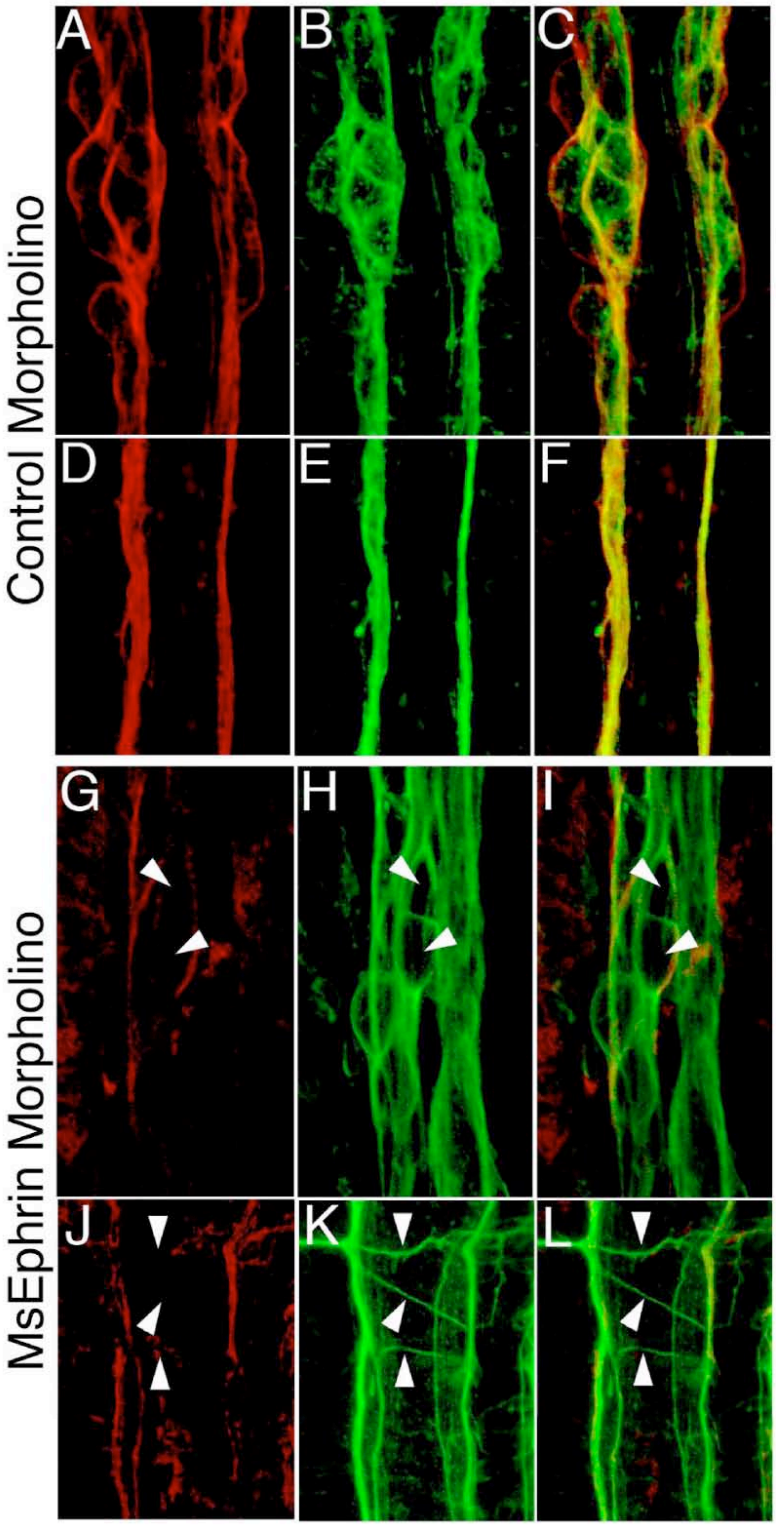


Figure 7.

Figure 7. Knock down of MsEphrin expression using antisense morpholinos results in midline crossover phenotypes. Cultured embryos were treated with 50 M control morpholinos (*A-F*) or MsEphrin-specific morpholinos (*G-L*), then double-immunostained with MsEphrin antibodies (to show the residual levels of MsEphrin expression) and anti-MsFas II antibodies (to reveal the full extent of EP cell migration and outgrowth, and to provide an off-target control). (*A-F*): in preparations treated with control morpholinos, both the migratory EP cells (*A-C*) and their leading processes (*D-F*) continued to express normal levels of both MsEphrin (red) and MsFas II (green), and they showed no abnormal midline crossing. *(G-L)*: in preparations treated MsEphrin-specific morpholinos, both the migratory EP cells (*G-I*) and their leading processes (*J-L*) exhibited a marked reduction in MsEphrin expression (red) but no detectable change in MsFas II expression (green). In addition, an increased number of neurons and processes in these preparations (lacking MsEphrin) traveled inappropriately onto the midline (crossovers indicated by white arrowheads). Scale bar = 10 μm.

Figure 8. Quantification of the effects of morpholino treatments on MsEphrin expression and migratory behavior by the EP cells in cultured embyros. *A*: the relative levels of MsFas II immunofluorescence in preparations treated with 50 M control morpholinos (black histograms) or MsEphrin-specific morpholinos (grey histograms) were not significantly different in either the EP cell bodies or their growing axons. MsFas II-specific immunofluorescence was normalized to background fluorescence levels in each preparation (see Methods and Suppl. Figs. 4-5). *B:* relative levels of MsEphrin immunofluorescence were significantly reduced in preparations treated with 50 M MsEphrin morpholinos, compared to preparations treated with control morpholinos (~90% reduction in the EP cell bodies and ~45% reduction in their distal axons). *C*: treatment with different concentrations of MsEphrin-specific morpholinos (1-50 M) caused a dose-dependent increase in the number of midline crossover events (including crossovers by migrating EP cells and their growing processes), compared to preparations treated with control morpholinos. *D*: quantification of the total average distance traveled by the migrating EP cells and growing axons along their muscle band pathways (normalized to untreated controls). Neither control morpholinos nor MsEphrin-specific morpholinos inhibited migration and outgrowth, but the highest concentration of MsEphrin morpholinos caused a significant *increase* in migration distance. Morpholino concentrations in *C* and *D* are indicated in μM. 14 preparations were used for each treatment condition, and significant differences were calculated with Student's 2-tailed t-test; **$p<0.01$, ***$p<0.001$.

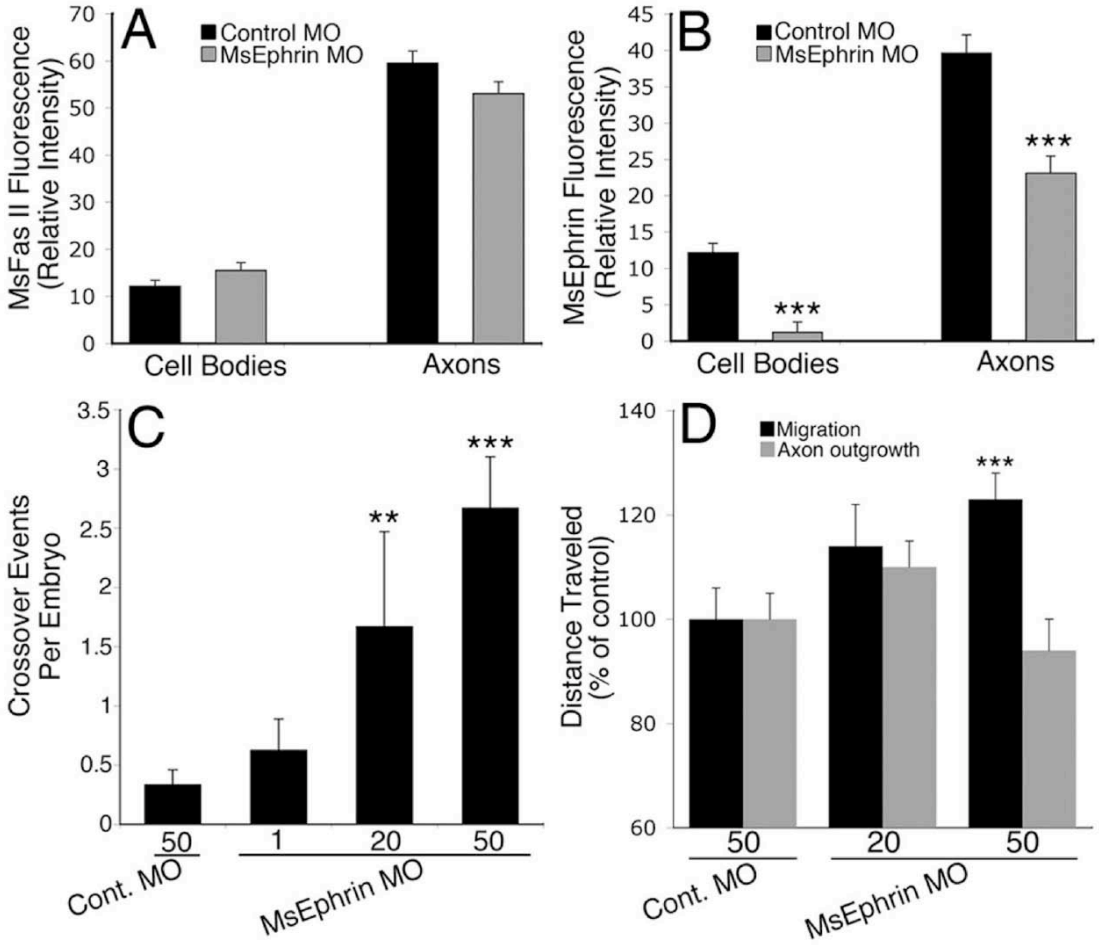

A
Control MO
MsEphrin MO
MsFas II Fluorescence (Relative Intensity)
70
60
50
40
30
20
10
0
Cell Bodies
Axons
B
Control MO
MsEphrin MO
MsEphrin Fluorescence (Relative Intensity)
45
40
35
30
25
20
15
10
5
0
***
***
Cell Bodies
Axons
C
Crossover Events Per Embryo
3.5
3
2.5
2
1.5
1
0.5
0
**
***
50
1
20
50
Cont. MO
MsEphrin MO
D
Migration
Axon outgrowth
Distance Traveled (% of control)
140
120
100
80
60
***
50
20
50
Cont. MO
MsEphrin MO

Figure 8.

DISCUSSION

The establishment of midline boundaries by Ephrin-Eph receptor interactions.

Graded distributions of Ephrins and Eph receptors help form topographic projection maps within the brain (Cheng et al., 1995; Drescher et al., 1997), but they also can be expressed in mutually exclusive patterns that define discrete tissue domains (e.g. (Cooke and Moens, 2002; Krull et al., 1997; Wang and Anderson, 1997; Xu et al., 1999). In many organisms, this process plays an important role in establishing midline boundaries. For example, during the formation of the vertebrate spinal cord, Ephrin-B3 is expressed by cells at the ventral midline that repel commissural neurons, in part via forward signaling through EphB receptors (Kadison et al., 2006). Multiple EphB receptors and Ephrin-B ligands have similarly been shown to regulate midline crossing by axons in the corpus callosum (Mendes et al., 2006). At the same time, interactions between several EphA receptors and their Ephrin-A ligands may attract a subset of callosal projections across the midline (Hu et al., 2003), although the extent to which cross-talk among the different Ephrin and Eph subtypes regulates neuronal guidance in this region remains poorly understood. Likewise in *Caenorhabditis elegans*, Eph-Ephrin interactions regulate axonal guidance at the ventral midline (Zallen et al., 1999), acting in conjunction with Ig-family receptors (Boulin et al., 2006).

Our results in *Manduca* have revealed a new example of this general theme, whereby spatially restricted patterns of a specific Ephrin ligand (MsEphrin) and its cognate Eph receptor (MsEph) help define an “enteric” midline during embryonic development. However, our data also provide strong evidence that reverse signaling

through a GPI-linked Ephrin is the primary mechanism by which cell-cell repulsion is regulated in this system. During normal ENS development, the EP cells migrate preferentially along the muscle band pathways of the gut (Fig. 9A) but also transiently

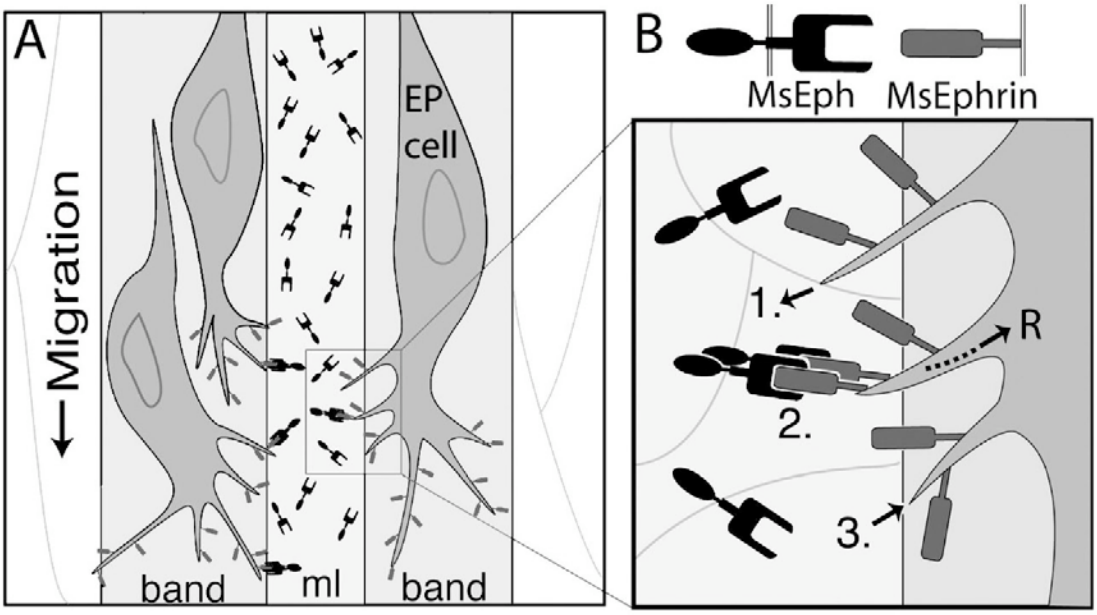


Figure 9. A model for the regulation of EP cell guidance by MsEphrin-MsEph receptor interactions at the enteric midline. *A*: during ENS development, the EP cells migrate and send axons posteriorly along the midgut muscle bands (**b**). Although they normally remain confined to the muscle bands, they also extend filopodia enriched in MsEphrin onto the adjacent interband regions; filopodia that extend onto the midline region (**ml**) encounter muscle cells that express the MsEph receptor. *B*: an enlarged view of the boxed region in A shows the hypothesized sequence of events that occurs at the midline. In step 1, an MsEphrin-positive filopodium extends onto the midline cells expressing MsEph receptors. In step 2, direct interactions between MsEph receptors and MsEphrin induces a reverse signaling response within the filopodium (R). In step 3, this interaction leads to the retraction of the filopodium off the midline cells (a repulsive response). Consequently, the EP cells and their growing processes are guided away from the midline, helping to maintain their correct trajectories along the band pathways. Other inhibitory cues (as yet unidentified) serve a similar function on the lateral interband regions. The mechanisms by which MsEphrin-MsEph complexes become uncoupled or cleared from the surface of the EP cell filopodia during their retraction remain to be determined.

extend processes onto the adjacent interband muscles and midline regions. When an MsEphrin-expressing filopodium from an EP cell extends onto the midline (Fig. 9B, step 1), it encounters MsEph receptors expressed by these cells (step 2), initiating a reverse signaling response via MsEphrin that results in filopodial retraction (step 3). In this manner, MsEph-MsEphrin interactions prevent inappropriate midline crossing by EP cells, maintaining the migratory neurons and their processes on the muscle band pathways.

In contrast to other preparations, where multiple Ephrins and Eph receptors are often expressed in overlapping patterns by the same cells (e.g. Frisen et al., 1999; Klein, 2001; Konstantinova et al., 2007), MsEphrin and MsEph are expressed in a strictly complementary pattern within the developing ENS: MsEphrin is only expressed by the migratory EP cells, while MsEph is only expressed by the midline muscle cells of the midgut (see also Fig. 1). Consistent with these expression patterns, MsEphrin fusion proteins bind specifically to the MsEph-expressing midline cells, while MsEph fusion proteins only label the MsEphrin-positive neurons (Figs. 3 & 5; see also Coate et al., 2007). Furthermore, our manipulations using cultured embryos suggest that reverse signaling via MsEphrin controls the behavior of the enteric neurons (summarized schematically in Supplemental Figure 6). First, targeting endogenous MsEph receptors on the midline cells with either monomeric MsEphrin-6His (Suppl. Fig. 6A) or dimeric MsEphrin-Fc proteins (Suppl. Fig. 6B) caused ectopic midline crossing by the EP cells, consistent with the model that both of these fusion proteins blocked endogenous MsEphrin-MsEph receptor interactions, thereby permitting the neurons to grow inappropriately across the midline. A similar crossover phenotype was produced by

targeting MsEphrin on the neurons with monomeric MsEph-6His (Suppl. Fig. 6C), a construct that should bind endogenous ligands without inducing reverse signaling events (c.f. (Davis et al., 1994). Thirdly, knocking down MsEphrin expression in the EP cells with morpholinos also induced these crossover phenotypes without inhibiting migration or outgrowth (Supp. Fig. 6D). Together, these results indicate that bioavailable MsEphrin ligands and MsEph receptors must both be present in the developing ENS to regulate the normal behavior of the migratory neurons at the midline.

In marked contrast to the foregoing experiments, treating the EP cells with dimeric MsEph-Fc constructs caused a significant *inhibition* of migration and outgrowth, as well as preventing midline crossing (Fig. 4 & 5; Suppl. Fig. 2). In short-term cultures, we found that MsEphrin-Fc treatments increased the number of filopodia extending from the leading processes of the neurons onto the midline (Fig. 6I), whereas MsEph-Fc caused a general reduction in filopodial number (Fig. 6I, J). Based on these findings, we propose that reverse signaling from MsEph receptors through MsEphrin on the migratory neurons normally prevents their leading processes from growing onto the midline regions, while hyperactivation of this signaling response (with dimeric MsEph-Fc) causes a general inhibition of their cell motility (Suppl. Fig. 6E).

Given the foregoing results, we were surprised that our short-term treatments with MsEph-Fc did not induce more dramatic changes in growth cone shape (Fig. 6K). Only when we applied MsEph-Fc at the onset of migration did we observe a global effect on EP cell motility, akin to the phenomenon of growth cone collapse *in vitro* (Evans et al., 2007; Harbott and Nobes, 2005). One explanation for this difference is that within the developing ENS, the EP cells may be most sensitive to MsEphrin-dependent signaling

during their initial stages of migration, when each neuron extends a wide array of exploratory filopodia before aligning with a particular band pathway (Copenhaver et al., 1996; Wright et al., 1999). Alternatively, additional guidance cues on the muscle bands (including MsFas II) may help stabilize the EP cells once they have begun to migrate, at which time MsEph-Fc dimers may only induce local changes in their leading processes without causing outright collapse.

Previous studies in cell culture have shown that dimeric MsEphrin-Fc constructs are also capable of activating *forward* signaling via MsEph receptors (Kaneko and Nighorn, 2003), consistent with the induction of forward signaling by Ephrins in vertebrates (e.g.(Davy and Soriano, 2005; Konstantinova et al., 2007; Yokoyama et al., 2001). An alternative explanation for our data might therefore involve a forward signaling response from MsEphrin on the EP cells via MsEph receptors on the midline cells, which in turn could induce a secondary feedback signal to induce filopodial retraction. However, by this scenario, applying MsEphrin-Fc complexes (targeting endogenous MsEph receptors at the midline) should *hyperactivate* this feedback signaling event, resulting in excessive filopodial retraction and a general inhibition of neuronal motility. Instead, these treatments induced the opposite effect, resulting in excessive midline crossing by both the neurons and their processes. Thus, simply occupying the ligand-binding sites of endogenous MsEph receptors with either monomeric (MsEphrin-6His) or dimeric constructs (MsEphrin-Fc) was apparently sufficient to induce ectopic midline crossing, regardless of MsEph receptor activation. Forward signaling through MsEph receptors might possibly regulate some later aspect of ENS development, although complete removal of the EP cells before migration produces no obvious defects

in midgut differentiation (Copenhaver et al., 1996). Therefore, our results support a role for reverse signaling but not forward signaling via MsEphrin in controlling neuronal growth and migration at the enteric midline.

An integrated response to multiple guidance cues regulates neuronal migration in the ENS.

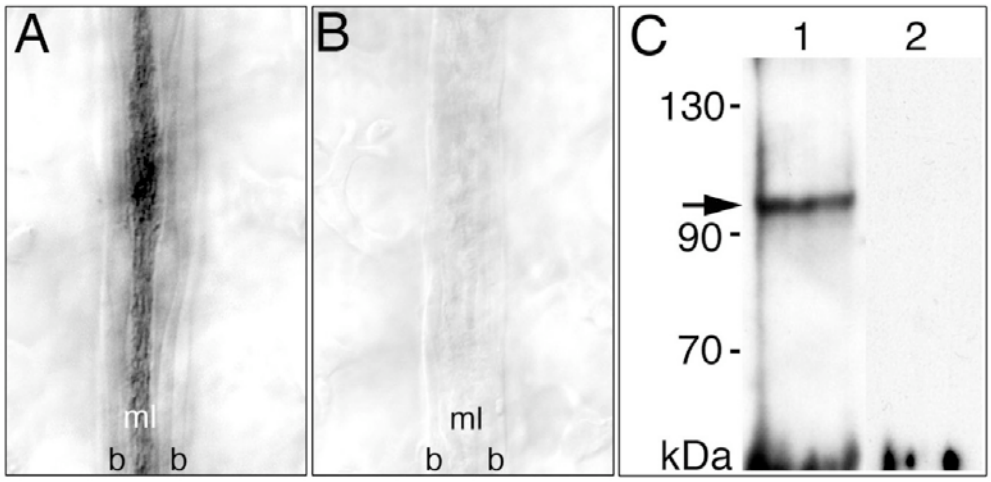
Previously, we showed that disrupting MsFas II-dependent interactions between the neurons and the muscle bands impeded their migration and outgrowth, indicating that this homophilic receptor promotes neuronal motility within the developing ENS (Copenhaver et al., 1996; Copenhaver and Taghert, 1989b). However, these manipulations did not induce ectopic growth onto the midline or the lateral interband regions, indicating the presence of other cues that prevent migration onto these non-pathway domains (Copenhaver, 2007; Wright and Copenhaver, 2000; Wright et al., 1999). Our current results indicate that MsEph receptors on the midline cells represent one of these inhibitory cues, signaling via MsEphrin on the neurons to prevent ectopic midline crossing. Intriguingly, both MsEphrin and MsFas II can be detected within the same populations of exploratory filopodia (Fig 1; and (Coate et al., 2007), suggesting that the net effects of attractive and repulsive guidance cues on local filopodial dynamics may ultimately determine the pathway chosen by each migratory neuron.

How might reverse signaling via MsEphrin modulate the behavior of migratory neurons, given that it is a GPI-linked ligand? Reverse signaling through type-B (transmembrane) Ephrins can be mediated via the activation of non-receptor Src family tyrosine kinases (SFKs; (Palmer et al., 2002), modulation of heterotrimeric G-proteins

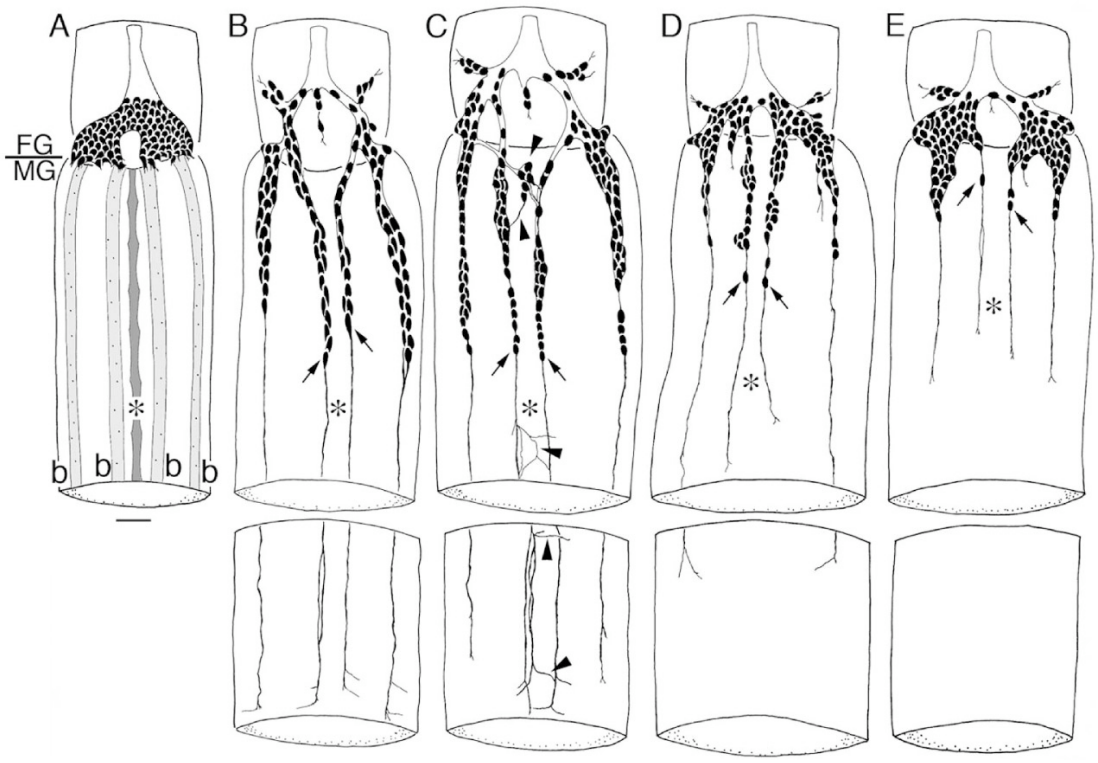
(Lu et al., 2001), or recruitment of other adapter and signaling molecules (Kullander and Klein, 2002). Reverse signaling via type-A (GPI-linked) Ephrins has remained more enigmatic, although examples of this process have now been implicated in the formation of retinotectal topographic maps (Knoll and Drescher, 2002) and the control of neurogenesis (Holmberg et al., 2005). Studies in cell culture have also indicated that reverse signaling by A-type Ephrins may involve the activation of non-receptor tyrosine kinases, which in turn can regulate integrin-dependent adhesion (Davy et al., 1999; Davy and Soriano, 2005; Huai and Drescher, 2001). By exploiting the accessibility of the developing ENS in *Manduca,* we can now investigate the mechanisms by which reverse signaling via a specific GPI-linked Ephrin (MsEphrin) regulates cellular motility *in vivo*, and how these signaling events are integrated with input from other guidance cues to direct neuronal migration within the developing embryo.

ACKNOWLEDGEMENTS

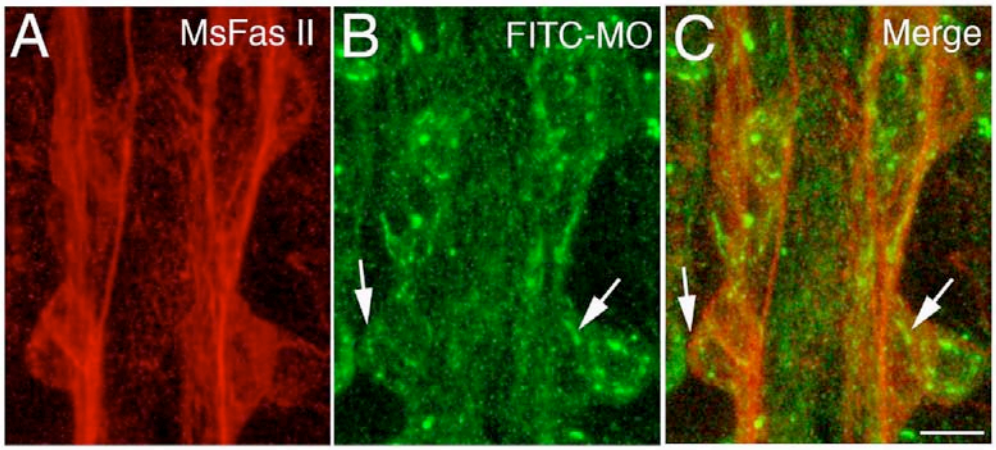
We thank Drs. Doris Kretzschmar and David Morton for their critical input on this manuscript. We are grateful to Drs. Michael Greenberg, Zak Wills, and Shannon Robichaud (Children's Hospital & Harvard Medical School) for generously providing their EphB2-specific antibodies and peptides. We also thank Drs. Ujwal Shinde and Hans-Peter Bächinger at OHSU for their assistance with the biophysical characterization of our fusion proteins. Lastly, we thank Ms. Tracy Swanson and Mr. Todd Vogt for their many helpful discussions relating to this work. This work was supported by NIH #AG025525 to PFC and by NIH Training Grant #T32 HD049309 to TMC.



Supplemental Figure 1. An anti-EphB2 antibody specifically recognize MsEph in *Manduca*. *A*: embryo at 60% of development that was immunostained with the anti-EphB2 antibody exhibited robust staining in the midline cells (ml), but not the adjacent muscle band pathways (b) or the EP cells (not visible). *B*: an identical preparation immunostained with an aliquot of the anti-EphB2 antibody that had been pre-adsorbed against its peptide epitope; all positive signal was eliminated. *C*: Western blot of protein lysate from *Manduca* pupal brain; staining with the anti-EphB2 antibody (lane 1) labeled a single 110 kDa band, corresponding to the appropriate size of the MsEph receptor (Kaneko & Nighorn, 2003). An identical blot stained with a pre-adsorbed aliquot of anti-EphB2 (lane 2).



Supplemental Figure 2. The effects of MsEphrin-Fc and MsEph-Fc treatments on EP cell migration and outgrowth; panels were re-drawn from *camera lucida* images of cultured embryos that had been fixed and immunostained with anti-MsFas II antibodies (compare with Figs. 3 & 5). *A*: embryo that was fixed at the onset of an experiment to illustrate the starting position of the EP cells at the foregut-midgut boundary (FG/MG); the muscle bands on the midgut that will be followed by the migratory EP cells are shown in stippled light gray, while the midline cells are illustrated in dark gray (and asterisk). *B*: embryo exposed to control Fc proteins exhibited the normal pattern and extent of migration and outgrowth along the muscle bands, with no signs of midline crossovers. *C*: embryo treated with 50 μg/ml MsEphrin-Fc showed a substantial number of midline crossovers by the EP cells and their processes (arrowheads), although the overall extent of migration and outgrowth along the muscle bands was similar to control. *D* and *E*: embryos treated with 20 μg/ml and 50 μg/ml MsEph-Fc, respectively; neither concentration induced an increase in midline crossovers, but MsEph-Fc did cause a concentration-dependent inhibition in the extent of EP cell migration and outgrowth along the muscle bands. Arrows indicate the leading EP cells on the mid-dorsal pair of muscle bands in each preparation. "b" = muscle band pathways in *A* (not shown in *B*-*E*); asterisks mark the position of the midline muscle cells. Scale bar = 30 μm.



Supplemental Figure 3. Treatment with CFSE-labeled morpholinos showed that morpholinos can be successfully introduced into migrating EP cells (as well as other cell types) in cultured embryos. *A-C*: a 2-μm confocal section from an embryo that was treated with 10 μM CFSE-labeled control morpholinos plus 0.6% Endo-Porter for 24 hr, and then fixed and immunostained with anti-MsFas II (to show the EP cells) and anti-FITC (which recognizes the CFSE tag). *A*: MsFas II labels the EP cell membranes and their processes; the brightly labeled, fasciculated axons from more anterior EP cell groups can be seen running between the neurons in this image. *B*: anti-FITC antibodies revealed the presence of CFSE-labeled morpholinos that were been internalized within the EP cells (white arrows indicate two of these neurons). *C*: merged image of *A* and *B*. Note that CFSE-labeled morpholinos could also be detected in other cell types on the gut surface, as expected with this method. However, neither Endo-Porter alone or Endo-Porter plus the control morpholino caused any defects in ENS development (as noted in Results). Scale bar = 10 μm.

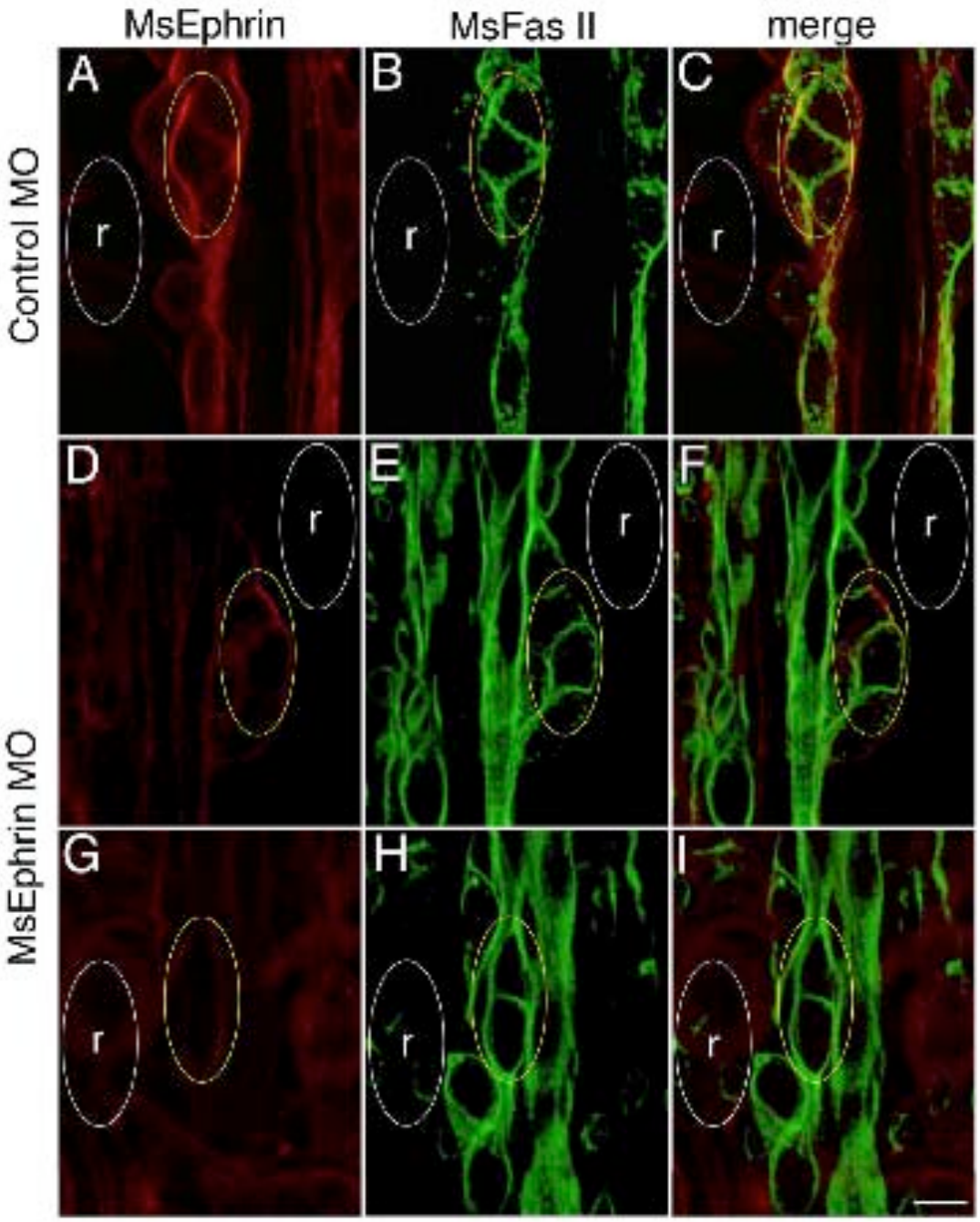


Figure S4

Supplemental Figure 4. Representative images illustrating the method used to quantify the relative levels of MsEphrin- and MsFas II-associated immunofluorescence in the EP cells of morpholino-treated preparations. A 2-μm Z-stack image (consisting of 4 X 0.5 μm sequential confocal sections) was used for the analysis of each preparation. The yellow ovals over a subset of EP cells in each preparation indicate the regions where mean levels of anti-MsEphrin immunofluorescence (using Cy3-conjugated secondary antibodies) and anti-MsFas II immunofluorescence (using Alexa 488-conjugated secondary antibodies) were measured, using ImageJ. The white ovals indicate the reference ("r") regions used to measure background fluorescent intensities for both fluorochromes. Relative fluorescent intensities were determined by subtracting the mean background levels from the mean fluorescent levels in each wavelength independently. *A-C*: example of a preparation treated with control morpholinos (50 M), in which MsEphrin and MsFas II expression in the EP cells appeared unaffected by the treatment. *D-F* & *G-I*: two examples of preparations that were treated with MsEphrin-specific morpholinos (50 M); in *D-F*, MsEphrin levels in the EP cells appeared substantially reduced (compared to controls); in *G-I*, MsEphrin levels were reduced below the sensitivity of our imaging methods. In contrast, MsFas II levels appeared unaffected in these preparations. Scale bar = 15 μm.

Supplemental Figure 5. Representative images illustrating the method used to quantify the relative levels of MsEphrin- and MsFas II immunofluorescence associated with the EP cell axons in morpholino-treated preparations. A 2-μm Z-stack image (consisting of 4 X 0.5 μm sequential confocal sections) was used for the analysis of each preparation. The yellow bars in each preparation indicate axonal regions where mean levels of anti-MsEphrin immunofluorescence (using Cy3-conjugated secondary antibodies) and anti-MsFas II immunofluorescence (using Alexa 488-conjugated secondary antibodies) were measured, using ImageJ. The white bars indicate the reference ("r") regions used to measure background fluorescent intensities for both fluorochromes. Relative fluorescent intensities were determined by subtracting the mean background levels from the mean fluorescent levels in each wavelength independently. *A-C*: example of a preparation treated with control morpholinos (50 μM), in which MsEphrin and MsFas II expression in the EP cells appeared unaffected by the treatment. *D-F* & *G-I*: two examples of preparations that were treated with MsEphrin-specific morpholinos (50 μM); in both preparations, MsEphrin levels in the EP cell axons were reduced compared to controls, although not as effectively as in the EP cell somata (compare with Fig. 7 and Suppl. Fig. 4). In contrast, MsFas II levels appeared unaffected in these preparations. Scale bar = 15 μm.

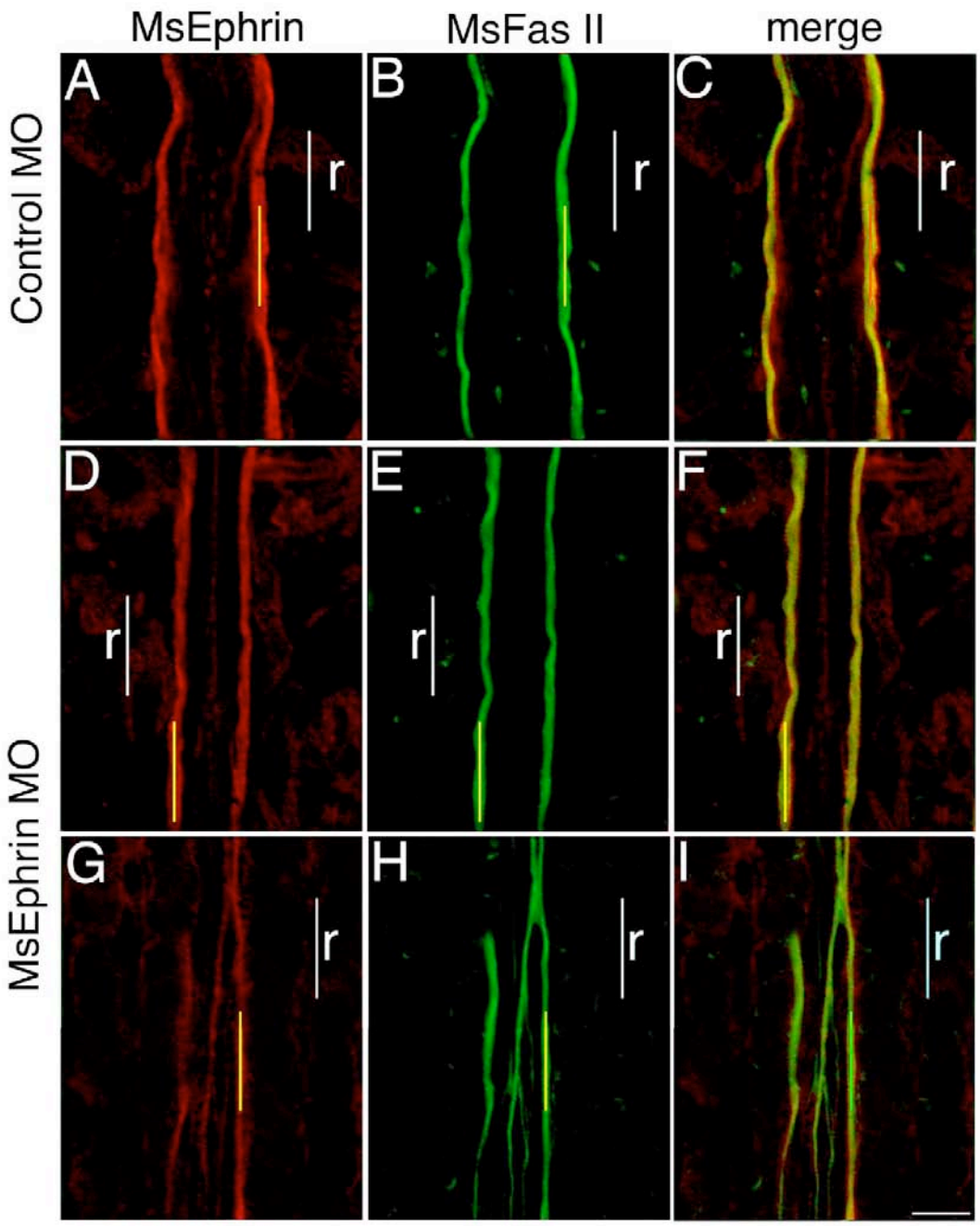


Figure S5

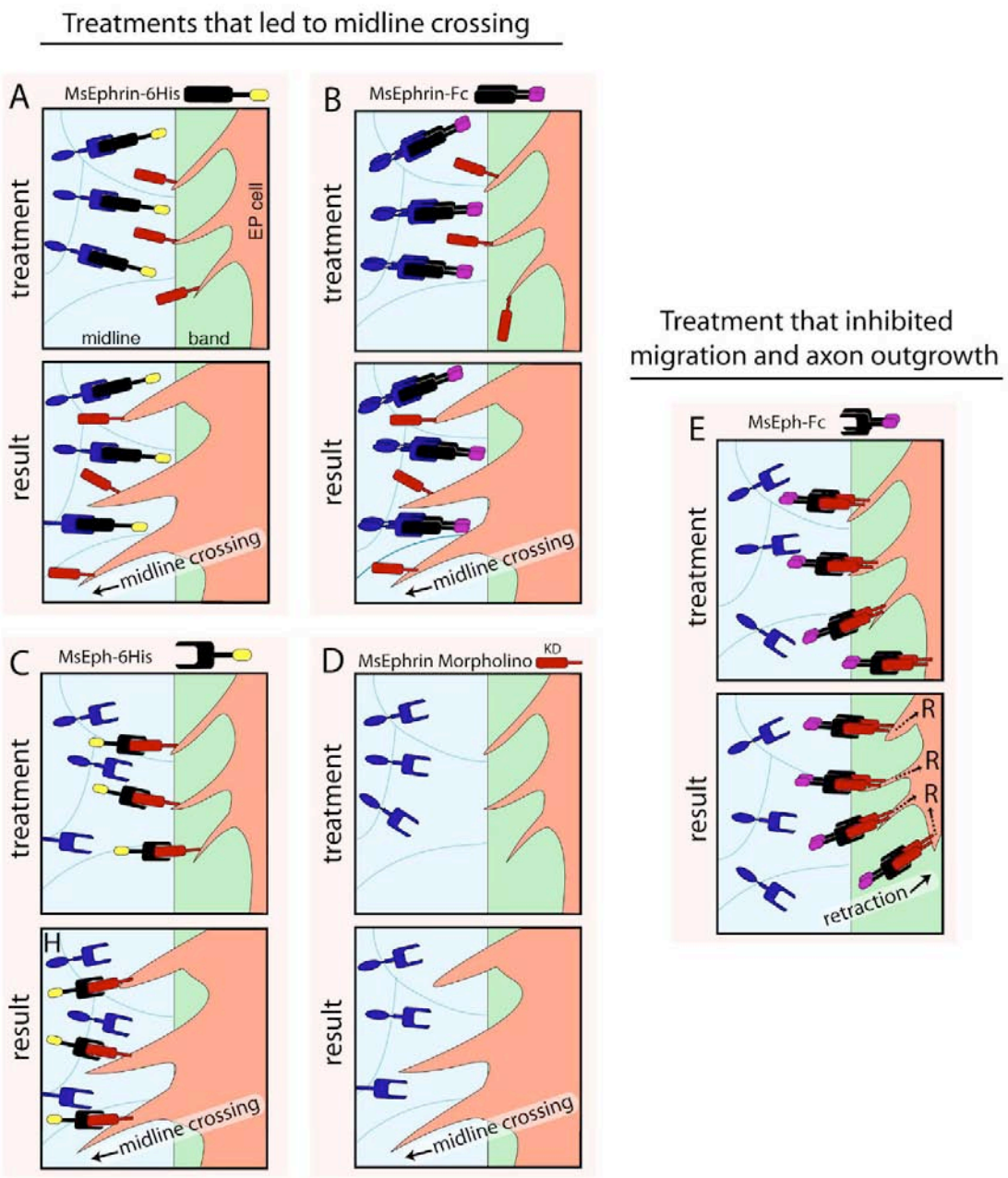

Treatments that led to midline crossing
A
MsEphrin-6His
treatment
EP cell
midline
band
result
midline crossing
B
MsEphrin-Fc
treatment
result
midline crossing
C
MsEph-6His
treatment
result
midline crossing
D
MsEphrin Morpholino
KD
treatment
result
midline crossing
Treatment that inhibited migration and axon outgrowth
E
MsEph-Fc
treatment
result
R
R
R
retraction

Figure S6

Supplemental Figure 6. Schematic summary illustrating the different manipulations used to test the role of MsEphrin-MsEph interactions in the developing ENS. In each set of panels, MsEphrin on the EP cell (pink) is illustrated in red, while MsEph receptors on the midline cells (light blue) is illustrated in dark blue. Panels *A-D* show treatments that caused midline crossovers, while Panel *E* shows the one treatment that caused an inhibition of EP cell migration and outgrowth. *A, upper panel*: monomeric MsEphrin-6His proteins are predicted to bind bioavailable MsEph receptors on the midline cells, thereby blocking interactions between endogenous MsEphrin on the neurons and the MsEph receptors without stimulating forward signaling. *A, lower panel*: MsEphrin-6His treatments led to midline crossovers by the EP cells and their processes, consistent with a blocking effect. *B, upper panel*: dimeric MsEphrin-Fc proteins are also predicted to bind bioavailable MsEph receptors on the midline cells, thereby blocking interactions between endogenous MsEphrin on the neurons and the MsEph receptors as well as potentially inducing forward signaling in the midline cells. *B, lower panel*: MsEphrin-Fc treatments led to midline crossovers by the EP cells and their processes, consistent with a blocking effect; forward signaling through the MsEph receptors does not appear to affect the behavior of the EP cells (which would be predicted to inhibit midline crossing). *C, upper panel*: monomeric MsEph-6His proteins are predicted to bind bioavailable MsEphrin ligands on the EP cells, thereby blocking interactions between endogenous MsEphrin and MsEph receptors on the midline cells without stimulating reverse signaling. *C, lower panel*: MsEph-6His treatments led to midline crossovers by the EP cells and their processes, consistent with a blocking effect. *D, upper panel*: knocking down MsEphrin expression in the EP cells with MsEphrin-specific morpholinos is predicted to prevent the EP cells from detecting MsEph receptors at the midline. *D, lower panel*: treatment with MsEphrin-specific morpholinos led to midline crossovers by the EP cells and their processes, accompanied by a loss of MsEphrin expression (see Fig. 7). *E, upper panel*: dimeric MsEph-Fc proteins are predicted to bind bioavailable MsEphrin ligands on the EP cells, thereby blocking interactions between endogenous MsEphrin and the MsEph receptors on the midline cells and potentially inducing reverse signaling in the EP cells. *E, lower panel*: MsEph-Fc treatments caused a reduction in filopodia extended by the EP cells in short-term cultures (Fig. 6), and an inhibiton in overall EP cell migration and outgrowth in long-term cultures (Fig. 4-5), suggesting that MsEph-Fc hyperactivated a reverse signaling response (R) in the EP cells. The molecular basis of this reverse signal is currently under investigation.

CHAPTER 3

A GPI-linked Ephrin regulates neuronal migration in Manduca via the local activation of a Src family kinase

Manuscript in preparation

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Tracy Swanson and Todd Vogt cloned MsSrc42 and MsSrc64.

Tracy Swanson performed the RT-PCR in figure 2.

ABSTRACT

We have investigated the extent to which reverse signaling via a GPI-linked Ephrin modulates the activity of a Src family kinase in order to guide motile neurons *in vivo*. We previously demonstrated that reverse (and not forward) signaling resulting from the activation of a GPI-linked Ephrin ligand in *Manduca* was required to prevent migratory neurons (the EP cells) from crossing the enteric midline. In this report, we have demonstrated that MsEphrin colocalizes with phosphorylated Src during periods of active EP cell motility. Pharmacological inhibition of Src *in vivo* resulted in aberrant midline crossing of the neurons – a phenotype that also results from inhibiting reverse signaling. Hyperactivating MsEphrin-mediated reverse signaling, by applying MsEph-Fc to explanted EP cells *in vitro*, and to cultured embryos *in vivo*, dramatically increased the phosphorylation state of Src. As demonstrated previously, culturing the EP cells in the presence of MsEph-Fc inhibited migration and outgrowth, but importantly, PP2 rescued this inhibitory effect. The results in the forthcoming report indicate that a Src family kinase is activated downstream of MsEphrin-mediated reverse signaling, an event that is necessary for appropriate midline guidance.

INTRODUCTION

During the formation of the nervous system, interactions between Eph receptors and their Ephrin ligands regulate multiple aspects of cell growth, migration and differentiation (Flanagan and Vanderhaeghen, 1998; Kullander and Klein, 2002; Pasquale, 2005; Wilkinson, 2001). In vertebrates, Eph receptors constitute the largest known family of receptor tyrosine kinases and are classified into two groups, based on their preferential affinities for different subclasses of Ephrin ligands. The EphA receptor group preferentially binds type A Ephrins, which are attached to the plasma membrane via glycosylphosphatidyl inositol (GPI) anchors, while EphB receptors typically bind type-B Ephrins, which are transmembrane proteins (Pasquale, 2004; Pasquale, 2005). However, in vivo analyses of their normal functions have been complicated by the discovery that Ephrin-Eph receptor interactions can be promiscuous both within and between subclasses, and multiple ligand-receptor combinations are often expressed by adjacent cells (Himanen et al., 2004). In addition, while Ephrin ligands can stimulate "forward" signaling by activating Eph receptors, Eph receptors can also stimulate "reverse" signaling in cells expressing their cognate Ephrin ligands, and the relative contributions of forward versus reverse signaling varies substantially, depending on the developmental context (Egea et al., 2005; Kullander and Klein, 2002). Reverse signaling via transmembrane Ephrin-B ligands contributes to a variety of morphogenetic events, including segmentation, axonal guidance, and synaptogenesis (Davy and Soriano, 2005; Wilkinson, 2001). Often, this process requires the activation of Src family kinases (SFKs), although a variety of other signaling cascades may also be modulated in response to Ephrin-B activation (Kullander and Klein, 2002). Intriguing examples of reverse

signaling via GPI-linked Ephrin-A ligands have now also been documented (Davy and Robbins, 2000; Holmberg et al., 2005; Huai and Drescher, 2001), although the mechanisms associated with this process are not well understood (Egea and Klein, 2007; Goldshmit et al., 2006; Kuijper et al., 2007).

Evidence that reverse signaling via Ephrin-A ligands serves important developmental functions has gradually emerged from several different lines of investigation. During the establishment of topographical projections in the vomeronasal system of the mouse brain, several Ephrin-As are expressed by ingrowing axons, while neurons in the accessory olfactory bulb express EphA receptors (Knoll et al., 2001a). Using a combination of genetic manipulations and in vitro stripe assays, Knoll et al. showed that interactions between Ephrin-As on the vomeronasal axons and EphA receptors in their target regions help guide the vomeronasal axons to their appropriate target regions. These findings contrasted with repellent interactions mediated by Ephrins and Eph receptors in other contexts, and strongly suggested this role in attraction was mediated by reverse signaling through these GPI-linked ligands (Knoll and Drescher, 2002; Knoll et al., 2001b). More recently, reverse signaling via Ephrin-A2 was shown to suppress neural progenitor proliferation within a presumptive stem cell niche in the adult mouse brain, where EphA receptors appeared to function primarily as ligands (Holmberg et al., 2005). Paradoxically, Ephrin-A-EphA signaling in embryonic cortical progenitors promotes apopotosis without affecting proliferation per se, further demonstrating the pleitrophic roles that these ligand-receptor interactions can play under different circumstances (Depaepe et al., 2005). In vitro studies (primarily using transfected cell lines) have also shown that reverse signaling via Ephrin-A5 can upregulate the presence

of phosphorylated Fyn (along with several other unidentified proteins), which in turn stimulates integrin-dependent adhesion (Davy et al., 1999; Davy and Robbins, 2000; Huai and Drescher, 2001). Reverse signaling via mutiple Ephrin-As in insulinoma cells was also shown to activate the small GTP-binding protein Rac and destabilize F-actin, leading to enhanced insulin secretion (Konstantinova et al., 2007). However, the lack of simple model preparations has hindered an analysis of how GPI-linked Ephrins can promote reverse signaling events under physiological circumstances (Egea and Klein, 2007; Goldshmit et al., 2006; Holmberg et al., 2005; Pasquale, 2005).

Recently, we reported a novel example of reverse signaling by a GPI-linked Ephrin in the developing enteric nervous system (ENS) of the tobacco hornworm *Manduca sexta* (Lepidoptera; Sphingidae; Linnaeus 1763). As in other insects, *Manduca* expresses only one Ephrin (MsEphrin, a GPI-linked ligand) and one corresponding Eph receptor (MsEph), greatly simplifying investigations into their normal functions (Coate et al., 2007; Kaneko and Nighorn, 2003). During the formation of the ENS, a population of 300 enteric neurons ("Enteric Plexus" or EP cells) must migrate and extend their axons along a set of pre-formed muscle bands on the midgut, without straying onto the adjacent interband musculature (Fig. 1A-C) (Copenhaver et al., 1996; Copenhaver and Taghert, 1989b). As they migrate, the EP cells express MsEphrin, and avoid midline cells that express MsEph receptors (Fig. 1D-E) (Coate et al., 2007). In a series of manipulations performed in cultured embryos, we showed that endogenous MsEphrin-MsEph receptor interactions prevent the neurons from growing across the enteric midline (Coate et al., 2008). Most notably, we showed that this response is mediated by *reverse* signaling through MsEphrin, whereas forward signaling via MsEph receptors on the midline

muscles played no apparent role in guiding the EP cells. In addition, we presented evidence that this repulsive effect is most likely mediated by the retraction of MsEphrin-expressing filopodia from points of contact with MsEph receptors at the midline, thereby steering the migrating neurons away from this inhibitory domain (Fig. 1F). However, the signaling mechanisms by which MsEphrin stimulation leads to this repulsive response remained undefined.

In this report, we have explored the role of SFKs in mediating reverse signaling by MsEphrin within the developing ENS. We present evidence that a *Manduca* orthologue of Src plays an essential role in this process, transducing the activation of MsEphrin by MsEph receptors into a retraction response by the migrating neurons away from the midline, thereby maintaining appropriate growth along their migratory pathways. These results also demonstrate an unusual example of Src-dependent control of motility, whereby MsEphrin-dependent activation of Src leads to the local inhibition, rather than enhancement, of neuronal outgrowth.

MATERIALS AND METHODS

Cloning and characterization of Manduca *Src* orthologues.

Partial clones of sequences encoding the *Manduca* orthologues of *Drosophila* Src42 (Src oncogene at 42A; hereafter referred to as MsSrc42) and *Drosophila* Src64 (Src oncogene at 64B; hereafter referred to as MsSrc64) were obtained from embryonic cDNA at ~65% development, using degenerate primers against evolutionarily conserved domains of SFKs from other insect species. To align Src42-related sequences from other species, we used the following GenBank accession numbers: for *Drosophila* Src42, NP 476849; for *Anopheles gambiae*, XM 316335; for *Apis mellifera*, XP 396043; for *Tribolium castaneum*, XP 969129. For *Bombyx mori*, partial sequences were constructed from contig sequences identified in SilkBase (http://morus.ab.a.u-tokyo.ac.jp/cgi-bin/index.cgi). To obtain the cDNA sequence encoding MsSrc42, a partial clone was obtained by nested reverse transcriptase-polymerase chain reaction (RT-PCR) in a PTC-100 thermocycler from MJ Research (Waltham MA), using Biolase Red Taq (Bioline; Boston, MA) and degenerate primers targeting the following Src42-specific sequences: for the outer reaction, primers were designed against 5'-DTQGDWW (gayacncarggngaytggtgg) and 3'-QWLED (tcytcnaryttccaytg). The outer PCR was performed with an annealing temperature of 55^{o}C and 1.5 min elongation for 30 cycles. For the inner reaction, degenerate primers were designed against 5'-EAEPWYF (garggngarccntggyaytt) and 3'MTNAEV (acytcngcrttngtcat) and used with an incremental PCR program, with an initial annealing temperature of 56^{o}C, decreasing by 0.1^{o}C at each cycle for 40 cycles. The remaining 5' sequence was amplified from

embryonic cDNA using exact primers against the deduced 5' sequence for Bombyx mori Src42 (atggggaattgctttagtagc) and the MsSrc42 sequence (ttgtggcgactctcggagtcg). The remaining 3' sequence was isolated by 3' rapid amplification of cDNA ends RACE (Frohman et al., 1988).

To align Src64-related sequences from other species, we used the following GenBank accession numbers: for *Drosophila* Src64B, P00528; for *Anopheles gambiae*, XM 316537; for *Aedes aegypti*, XM 001649397; for *Apis mellifera*, XM 396908; for *Tribolium castaneum*, XM 970629. For *Bombyx mori*, partial sequences were constructed from contig sequences identified in SilkBase. To obtain the cDNA sequence for MsSrc64, nested RT-PCR was performed using embryonically derived cDNA and degenerate primers targeting the following evolutionarily conserved amino acid sequences: for the outer reaction, primers were designed against 5'VAIKTL (gtngcnathaaracnyt) and 3'WTAPEA (ngcytcnggngcngtcca); for the inner reaction, primers were designed against 5'AFLQEA (gcnttyytncargargc) and 3'DFGLAR (ckngcnarnccraartc). Incremental PCR programs were used with Platinum PCR Supermix (Invitrogen) for the outer reaction (initial annealing temperature = 55^{o}C, increased each cycle by 0.3^{o}C for 30 cycles) and inner reaction (initial annealing temperature = 58 C, increased each cycle by 0.1^{o}C – 0.1 C for 40 cycles). The remaining 5' and 3' ends were isolated by PCR from a phagemid library prepared from Manduca brain cDNA (in pBluescript II KS+; gift of Dr. James Nardi), using nested primer combinations derived from the initial MsSrc64 sequence and primers against flanking pBluescript II sequences (Stratagene).

Embryo preparation and immunostaining.

M. sexta embryos were collected from an in-house breeding colony and staged according to standard internal and external characteristics (1 hour at 25°C = 1% of development) (Copenhaver and Taghert, 1989b). All reagents are from Sigma Aldrich (St. Louis, MO), unless otherwise noted. Embryos were dissected in defined saline (140 mM NaCl, 5 mM KCl, 28 mM glucose, 40 mM $CaCl_2$, and 5 mM HEPES; pH 7.4; (Coate et al., 2008; Horgan and Copenhaver, 1998), then fixed in 4% paraformaldehyde (pH 7.4; Electron Microscopy Sciences, Hatfield, PA) for 1 h at room temperature (RT). Embryos were then rinsed and permeabilized with phosphate-buffered saline plus 0.6% Triton-X 100 (PBST), then incubated for at least 1 hr in PBST plus 10% normal serum and 0.1% NaN_3. For preparations probed with antibodies against phosphorylated forms of Src orthologues (tyrosine kinases homologous to the Rous sarcoma virus oncogene protein pp60(v-Src); see below), a 1% solution of bovine serum albumin (BSA, immunoanalytical grade; Promega; Madison, WI) was used instead of normal serum to avoid exposure to exogenous phosphatases (Garcia et al., 2004). Primary antibodies were then diluted in blocking solution and applied to the embryos for 2 hr at RT or overnight at 4°C at the following concentrations: mouse anti-pan fasciclin II (MsFas II; monoclonal C3), 1:20,000 (Wright et al., 1999); guinea pig anti-transmembrane Fas II (TM-MsFas II), 1:1000 (Wright and Copenhaver, 2000); mouse anti-Src, 1:1000 (7G9; Cell Signaling Technologies, Danvers, MA); mouse anti-phospho Src (pY416) (pSrc), 1:100-1:500 (Cell Signaling Technologies). To detect MsEphrin, unfixed embryos were incubated for 90 min at RT with anti-MsEphrin (1:100; (Coate et al., 2007; Coate et al., 2008), then post-fixed with 4% paraformaldehyde and labeled with fluorochrome-conjugated secondary

antibodies (for 1 hr at RT) (Coate et al., 2008). Embryos were then counterstained with additional primary and secondary antibodies and mounted in SlowFade Gold (Invitrogen). Alexa-conjugated secondary antibodies (Invitrogen) were used at 1:1000; Cy3- and Cy5-conjugated secondary antibodies (Jackson ImmunoResearch; West Grove, PA) were used at 1:400-1:800. Preparations were imaged on a BioRad 1024 ES laser scanning confocal microscope in the Live Cell Imaging Facility in the Center for Research on Occupational and Environmental Toxicology, OHSU, with the assistance of Dr. Stefanie Kaech-Petrie. Z-stack images were then flattened, pseudo-colored, and uniformly adjusted for brightness and contrast, as needed in Photoshop (Adobe Systems, San Jose, CA).

Immunoblots of embryonic tissues and GV1 cell lysates.

Tissues from staged embryos were dissected and frozen on dry ice, then homogenized in Laemmli buffer following our published methods (Swanson et al., 2005). Extracts were also prepared from cultures of Manduca GV1 cells (Hiruma and Riddiford, 2004). Protein extracts (25 μg per lane) were then separated by electrophoresis in 10% polyacrylamide gels under reducing conditions and transferred to nitrocellulose. Immunoblots were pre-blocked for 1 hr using Blotto B solution (Rockland Immunochemicals; Gilbertsville, PA), incubated with anti-Src (1:1000) or anti-pSrc (1:1000) antibodies overnight at 4^{o}C, then rinsed and incubated with secondary antibodies conjugated to horseradish peroxidase (HRP; Jackson ImmunoResearch). Bound antibody was visualized using WestPico chemiluminescence substrates (Pierce; Rockford, IL).

In vivo manipulations of cultured embryos.

For overnight cultures, embryos were isolated at 52-53% of development (prior to the onset of EP cell migration) in sterile embryonic culture medium (Horgan et al., 1995). Embryos were restrained in Sylgard-coated chambers, and the developing ENS was exposed by making a small incision in the dorsal body wall. The EP cells were then treated with culture medium containing the Src inhibitors PP2 (4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine; 1-100 μM) or SU6656 (10 μM; Calbiochem; Gibbstown, NJ), or with medium containing matched concentrations of DMSO (up to 0.1% v/v as a vehicle control). The embryos were allowed to develop for another 24 hr at 28°C (through phases of EP cell migration and outgrowth) and then re-dissected to expose the developed ENS. The preparations were then fixed and immunostained with anti-MsFas II antibodies to reveal the complete distribution of EP cells and their processes on the foregut and midgut. For these studies, the ABC-HRP kit (Vector Laboratories; Burlingame, CA) was used to detect MsFas II immunoreactivity, using diaminobenzidine with H_20_2 as a colorimetric substrate. The full extent of EP cell migration, axon outgrowth, and midline crossing events was quantified using *camera lucida* and photomicrographic techniques, as previously described (Coate et al., 2008; Wright et al., 1999). To stimulate reverse signaling via endogenous MsEphrin on the EP cells, MsEph-Fc fusion proteins were generated following our published methods (Coate et al., 2007; Coate et al., 2008) and applied to the developing ENS in the presence of 0.1% DMSO (to match our other experimental conditions).

Filopodial orientation assays were performed essentially as described (Coate et al., 2008). Briefly, the developing ENS was exposed in embryos at 60% of development

(a period of active EP cell outgrowth) and treated with μM PP2, 10 μM SU6656, or vehicle control for 3 hr at 28°C. The preparations were then fixed and immunostained with anti-MsFas II antibodies. For these studies, MsFas II immunostaining was detected with Alexa-fluor 488-conjugated secondary antibodies; some preparations were also immunostained with anti-pSrc (detected with anti-mouse Cy3-conjugated secondary antibodies). The preparations were then visualized by confocal microscopy, and compressed Z-stack images of the leading processes of the EP cells were analyzed to determine the percentage of their filopodia that had aberrantly extended onto the inhibitory midline regions of the gut. Morphometric measurements were performed as described previously (Coate et al., 2008).

Single gut explant assays.

Embryos at 60-62% of embryonic development were isolated and dissected in defined saline (supplemented with $CaCl_2$ and ~0.5% BSA). The intact midgut was explanted by carefully incising the surrounding trachea and adjacent regions of the foregut and hindgut. Using a micropipette, single embryonic midguts (~300 m long by 100 m diameter) were transferred into individual microcentrifuge tubes containing 10 μl of defined saline, plus either MsEph-Fc or MsEph-6His fusion proteins (25μg/ml), or human Fc as a control (Jackson ImmunoResearch). This concentration was chosen on the basis of our previous studies demonstrating the ability of MsEph-Fc to stimulate MsEphrin-dependent reverse signaling in cultured embryos (Coate et al., 2008). In some experiments, isolated midguts were pretreated with 10 μM PP2 or PP3 (4-amino-7-phenylpyrazolo[3,4-d]pyrimidine; a negative control for PP2; Calbiochem) for 20

minutes, prior to the addition of the MsEph fusion proteins. After 15-60 min incubation at RT, the samples were transferred onto dry ice to halt additional signaling activity. Individual midguts were then lysed and homogenized by adding 10 μl chilled 2X lysis buffer, and electrophoresed in separate lanes on 10% polyacrylamide gels. The samples were then transferred to nitrocellulose membranes, blocked using Blotto B solution, and probed with anti-pSrc antibodies (1:1000) overnight at 4°C. Secondary antibodies were applied and detected as described above.

In vivo Src activation, imaging, and quantification.

Embryos at 60-62% of development were dissected in defined saline and dissected to expose the developing ENS, as described above. The migrating EP cells were then treated with MsEph-Fc or control Fc proteins (25 μg/ml), and then the embryos were incubated for 25 minutes at RT. For some experiments, the preparations were pre-treated with PP2 or PP3 for 20 min, prior to the addition of the Fc fusion proteins. At the end of the incubation period, the embryos were fully dissected, fixed in chilled 4% paraformaldehyde for 1 h, then rinsed extensively in PBST. After pre-blocking for 2 hours in 1% BSA, they were then double-immunostained with anti-pSrc (1:500) and anti-MsFas II antibodies (1:2000), as described above. After extensive rinsing, fluorochrome-conjugated secondary antibodies were applied for exactly 1 hour. Confocal Z-stack images were then acquired in the region of the leading axons under linear parameters; identical laser and acquisition settings were used to image each preparation.

For quantifying fluorescence intensities associated with the EP cell processes, two of the eight Z-stack sections (encompassing the majority of the leading filopodia) were

flattened using MetaMorph Imaging software (Molecular Devices; Sunnyvale, CA), and brightness and contrast levels were adjusted uniformly in Photoshop. Relative immunofluorescent intensities were quantified for individual filopodia by tracing a line from its tip to its base at the body of the growth cone. For each condition, ≥10 growth cones and >100 filopodia were analyzed. Numerical values for fluorescence intensity were acquired using ImageJ software (http://rsb.info.nih.gov/ij/). Statistical analyses were performed using Students' 2-tailed t-test to compare means; sample sizes were based on the number of growth cones analyzed per condition.

RESULTS

Reverse signaling via MsEphrin regulates EP cell behavior at the enteric midline.

The formation of the ENS in *Manduca* involves a stereotyped sequence of neuronal migration by the EP cells, during which MsEph and MsEphrin are expressed in a strictly complementary pattern (Coate et al., 2007). By 55% of development, the EP cells (Fig. 1A; red cells) have delaminated from a neurogenic placode in the foregut epithelium and spread bilaterally around the foregut-midgut boundary, whereupon they each begin to align with one of eight longitudinal muscle bands that have coalesced on midgut surface (Fig. 1A; green; only the four dorsal muscle bands are shown). By 60% of development (Fig. 1B), subsets of EP cells have begun to migrate rapidly down each band pathway; during this migratory phase, the neurons also extends exploratory filopodia from their leading processes onto the lateral interband muscles and the midline interband regions (Fig. 1, blue cells), but strictly avoid growing onto these non-pathway domains (Copenhaver, 2007; Copenhaver and Taghert, 1989b). By 65% of development (Fig. 1C), the migratory dispersal of the EP cells is complete, but they continue to extend axons along the muscle bands for another 15% of development before eventually branching laterally to provide a diffuse innervation to the midgut musculature (Copenhaver and Taghert, 1989a; Copenhaver and Taghert, 1989b).

In previous studies, we showed that Ephrin-Eph receptor interactions restrict the EP cells from growing onto the midline regions of the gut. Specifically, we showed that the migratory neurons and their processes express MsEphrin (Fig. 1D-E, red), while the midline muscle cells express the cognate Eph receptor (MsEph; Fig. 1D-E, blue) (Coate

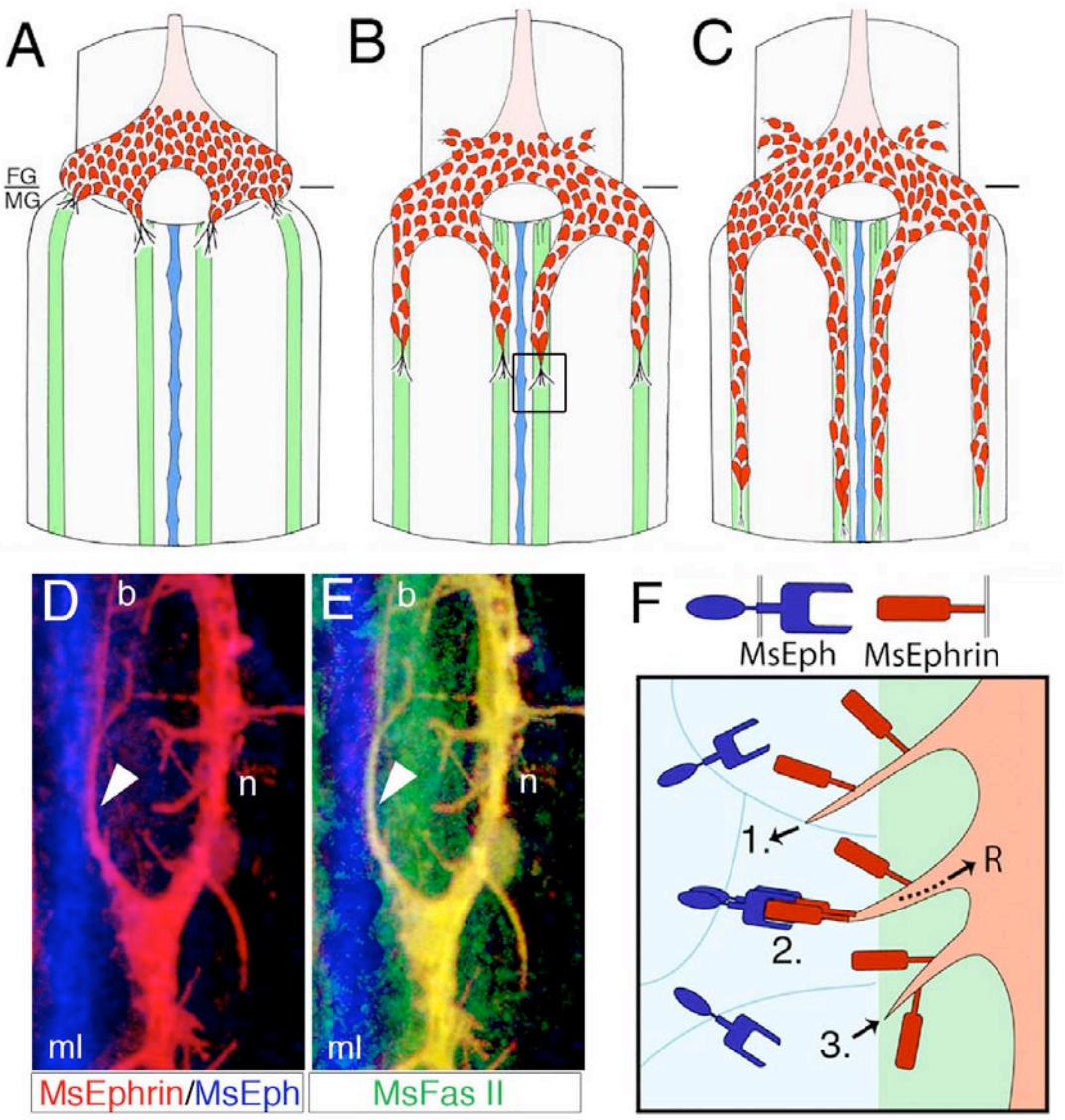
A
B
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Figure 1.

Figure 1. MsEphrin and MsEph are expressed in distinct domains during ENS formation in *Manduca sexta*. *A-C*: a schematic view of the migratory behavior of neurons that form the enteric plexus ("EP cells"; red). *A*: at ~55% of development, all of the EP cells express MsEphrin, many of which have begun to align with each of eight muscle band pathways (green), which express MsFas II (the EP cells also express MsFas II; not illustrated in *A-C*). Only the four dorsal muscle bands are shown. *B*: at ~60% of development, the EP cells migrate posteriorly along the muscle bands while avoiding the midline muscle cells (blue), which express MsEph. Some of the EP cells begin migrating anteriorly into the foregut region. *C*: at ~65% of development, the EP cells have completed migration, but they continue to extend axonal processes along the band pathways while avoiding the midline where MsEph is expressed. FG/MG = the foregut-midgut boundary. *D-E*: an immunostained embryo at 63% of development approximately representing the boxed region in B. *D*: the leading processes of the neurons (n) express MsEphrin (red). MsEphrin is enriched within the filopodial processes (see arrowhead) that freely explore the muscle band pathways (b), but do not cross the midline (ml) where MsEph is expressed (blue). *E:* the same embryo as in D, but counterstained with MsFas II antibodies to label the band pathways (b) and the neurons (n). The midline muscle cells (ml) do not express MsFas II. *F*: a schematic representation of the previous model whereby MsEphrin-MsEph interactions stimulate reverse signaling to confer cell-cell repulsion at the midline. (Step 1) filopodia expressing MsEphrin ligands (red) protrude into the midline where MsEph receptors are expressed (blue). (Step 2) the transient engagement of MsEph and MsEphrin stimulates a reverse signal (R) to steer the filopodium away from the midline (step 3). This paper investigates the mechanism of MsEphrin-mediated reverse signaling (R). Scale bar in E = 5 μm.

et al., 2007). Notably, we observed MsEphrin-positive filopodia extending from the EP cells up to, but not across, the MsEph-expressing midline cells (Fig. 1-D), suggesting that interactions between the ligand and receptor control the motile behavior of the neurons at the midline. In a series of manipulations performed in cultured embryos, we subsequently showed that endogenous MsEphrin-MsEph interactions are indeed responsible for preventing the neurons and their processes from crossing the midline inappropriately (Coate et al., 2008), a theme that has been reiterated at the midline regions of a variety of organisms. More unusual was our discovery that within the developing ENS of Manduca, this process is mediated by *reverse* signaling through

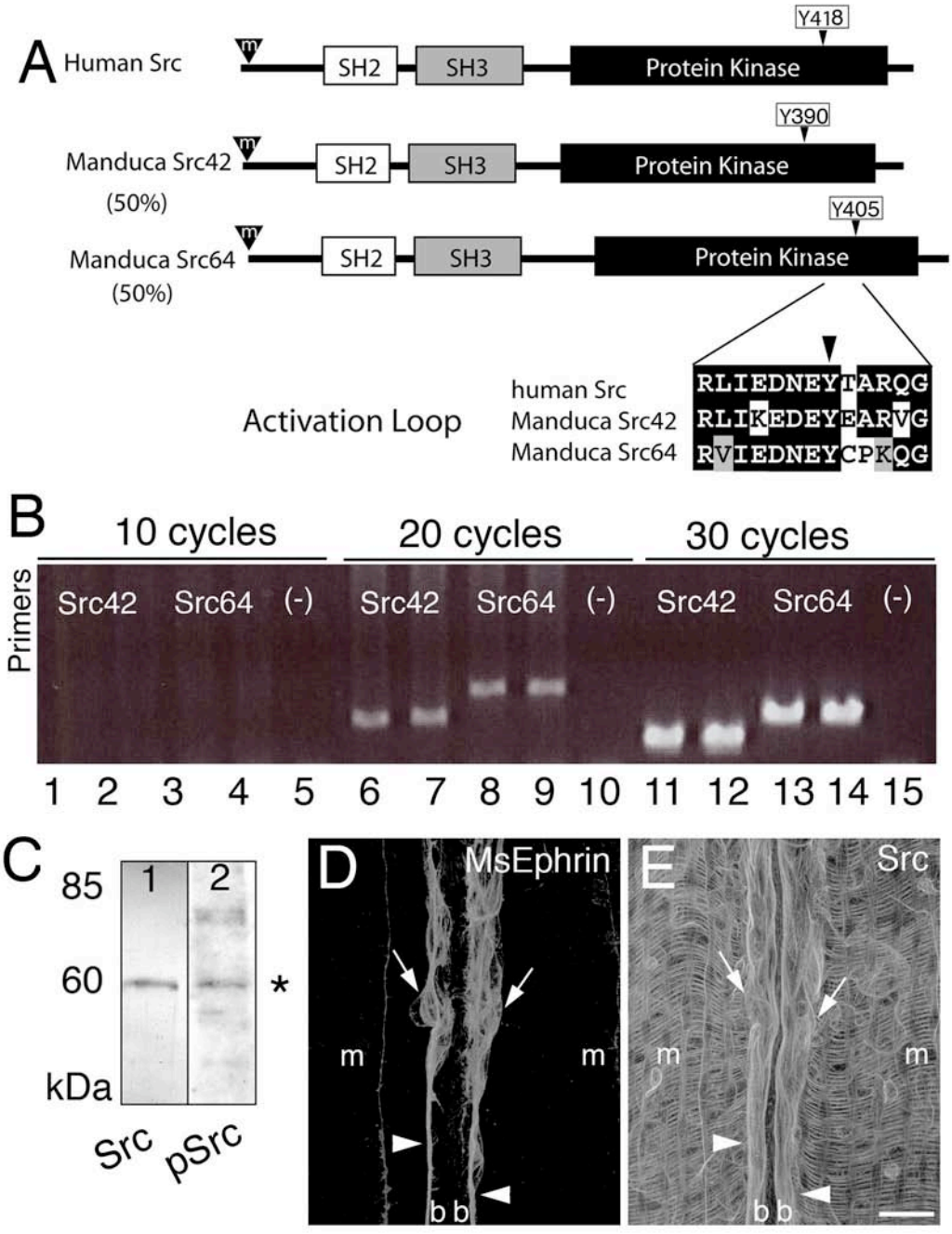
MsEphrin on the neurons, whereas *forward* signaling via MsEph receptors on the midline cells played no apparent role in controlling the behavior of the neurons or their processes. These results support the model (Fig. 1F) that MsEph receptors on the midline cells simply activate a reverse signaling response via MsEphrin on filopodia that are extended onto these regions, leading to a retraction response that helps restrict the migrating neurons and their growing axons to their normal muscle band pathways. This preparation thus provides the first unambiguous example of reverse signaling via a GPI-linked Ephrin ligand in the control of neuronal guidance in vivo; however, the signal transduction mechanisms by which activated MsEphrin ligands mediate this repulsive response remained unexplored (Fig. 1F, 'R').

Isolation of two *Manduca* Src orthologues as candidate mediators of MsEphrin reverse signaling.

As noted above, previous studies in cell culture have indicated that SFKs may participate in reverse signaling events triggered by type-A (GPI-linked) Ephrins (Davy et al., 1999; Huai and Drescher, 2001). However, attempts to identify the specific tyrosine kinases involved in this process have produced conflicting results, possibly due to the expression of multiple SFKS with overlapping functions in different vertebrate cell types (Gauld and Cambier, 2004; Parsons and Parsons, 2004; Thomas and Brugge, 1997). In contrast, only two SFKs are expressed in *Drosophila* (Simon et al., 1985; Takahashi et al., 1996): Src42A (Src oncogene at chromosome position 42A), which is the closest orthologue to vertebrate Src; and Src64B (Src oncogene at chromosome position 64B), a second Src orthologue that is unique to insects (Kussick and Cooper, 1992; Simon et al.,

1985). Src64B is required for several aspects of oogenesis (Dodson et al., 1998; O'Reilly et al., 2006), while Src42A selectively regulates adherens junction stability in tracheal epithelia (Shindo et al., 2008), but both protein are widely expressed in the developing nervous system (Simon et al., 1985; Takahashi et al., 2005) and might potentially play a role in Ephrin-dependent reverse signaling.

Accordingly, we isolated full-length clones encoding the *Manduca* orthologues of Src42A (hereafter designated MsSrc42A) and Src64B (designated MsSrc64B). Both proteins have predicted molecular sizes of ~60-62 kDa, similar to the size of SFKs in other species (Takahashi et al., 1996; Thomas and Brugge, 1997). MsSrc42A shares 83% identity with Drosophila Src42A, and 56% identity with both human c-Src and Fyn. MsSrc64B shares 77% identity with *Drosophila* Src64B and ~45% identity with both human SFKs. Like their counterparts in *Drosophila,* MsSrc42A and MsSrc64B contain all of the hallmark structural features that distinguish prototypical Src family kinases (Fig. 2A), including myristylation consensus sites in the SH4 N-terminal domain (at glycine 2; 'm'), SH3 and SH2 domains, and a C-terminal tyrosine kinase domain (Boggon and Eck, 2004; Roskoski, 2004). Both *Manduca* proteins also possess a conserved activation loop within their catalytic domains, containing tyrosine residues (Y390 in MsSrc42A; Y405 in MsSrc64B; Fig. 2A) that correspond to the autophosphorylation site in human c-Src (Y418) (Boggon and Eck, 2004; Parsons and Parsons, 2004; Roskoski, 2004). Likewise, conserved tyrosine residues corresponding to the site of inhibitory phosphorylation in human c-Src (Y527) are located near the C-terminal end of the catalytic domain (Y500 in MsSrc42A; Y518 in MsSrc64B).


A
Human Src
SH2
SH3
Protein Kinase
Y418
Manduca Src42
(50%)
Y390
Manduca Src64
(50%)
Y405
Activation Loop
human Src
Manduca Src42
Manduca Src64
RLIEDNEYTARQG
RLIKEDEYEARVG
RVIEDNEYCPKQG
B
10 cycles
20 cycles
30 cycles
Primers
Src42
Src64
(-)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
C
85
60
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1
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*
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MsEphrin
E
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Figure 2.

Figure 2. Two *Manduca* Src kinase orthologues are expressed during embryonic development. *A*: a schematic comparison of MsSrc42 and MsSrc64 with human c-Src (not including all known structural features). Both *Manduca* Src orthologues contain N-terminal myristylation consensus sites, SH2 and SH3 protein-protein interaction domains and a large C-terminal protein tyrosine kinase domain. High conservation within the activation loop (see highlighted sequence) is observed: Y390 in MsSrc42 and Y405 in MsSrc64 are likely tyrosine phosphorylated (see arrowhead) when their parent proteins are activated. *B*: RT-PCR using gene-specific primers shows that MsSrc42 and MsSrc64 are expressed at relatively equal levels in the embryo. Embryonic cDNA was used as a template and 10 (lanes 1-5), 20 (lanes 6-10) and 30 (lanes 11-15) cycles were run to try and distinguish varying levels of MsSrc42 or MsSrc64 mRNA. (-) represents negative control reactions that contained no cDNA. We observed no differences in transcript levels between MsSrc42 and MsSrc64 in all cycle sets. *C*: a Western blot showing the identification of a ~60 kDa species likely representing Src (lane 1) and pSrc Y416 (lane 2) from *Manduca* GV1 cell lysates. Both antibodies target the amino acid sequence within the putative Src activation loop (highlighted in A). *D-E*: non-phospho-Src is detectable in nearly all cells along the *Manduca* gut, including the EP cells. *D*: MsEphrin is expressed in the soma of the EP cells (arrows) and their leading processes (arrowheads), but not along the band pathways (b) or the adjacent musculature (m). *E*: in contrast, Src appears to be expressed in all cell types examined.

In Drosophila, Src64B has been shown to play an essential function in ring canal development during oogenesis (Dodson et al., 1998; Roulier et al., 1998), while Src42A is required for more widespread signaling activities (Laberge et al., 2005; Lu and Li, 1999). To gauge the relative expression levels of MsSrc42A and MsSrc64B during embryonic development, we performed RT-PCR using embryonic cDNA and primer sets specific for the coding domain of each Manduca Src orthologue. As shown in Figure 2B, mRNA sequences encoding both proteins are expressed at similar levels, suggesting that, as in flies, both proteins have a relatively widespread distribution in developing embryos.

Using commercial antibodies that recognize conserved domains shared by both vertebrate and invertebrate Src orthologues, we found that both anti-Src and anti-pSrc antibodies labeled a ~60 kDa protein band in immunoblots of Manduca GV1 cell lysates (Fig. 2C), as well as in lysates derived from embryonic and larval sources (not shown).

Because these antibodies may recognize both MsSrc42A and MsSrc64B equally well, we hereafter will refer to their activity as detecting "Src-related" proteins in Manduca tissues. In the developing ENS, MsEphrin is selectively expressed by the EP cells (arrows) and their processes (**arrowheads**; Fig. 2D), as reported previously (Coate et al., 2007; Coate et al., 2008). By contrast, immunoreactivity was detectable within all cell types associated with the ENS, including the EP cells and their processes, the underlying band pathways (**b**), and the adjacent interband musculature (**m**).

MsEphrin and active Src co-localize during ENS development.

We hypothesized that if Src was associated with MsEphrin reverse signaling, then activated Src might preferentially co-localize with MsEphrin in the leading processes of the EP cells. We therefore double-labeled embryos with anti-MsEphrin and anti-pSrc antibodies at various times during EP cell migration and outgrowth. Strikingly, pSrc immunoreactivity was concentrated in the motile EP cells and showed considerable co-localization with MsEphrin (Fig. 3). During early periods of migration (56-58% of development), while the EP cells were beginning to travel onto the muscle band pathways (**b**), MsEphrin and pSrc immunostaining co-localized at the plasma membranes of the EP cell somata (Fig. 3A-C, arrows) and their exploratory filopodia associated with their leading processes (Fig. 3A, arrowheads). During the subsequent period of axon outgrowth by the post-migratory EP cells (65-80%), robust levels of MsEphrin and pSrc immunoreactivity continued to be detected in their fasciculated axons (Fig. 3G-F,

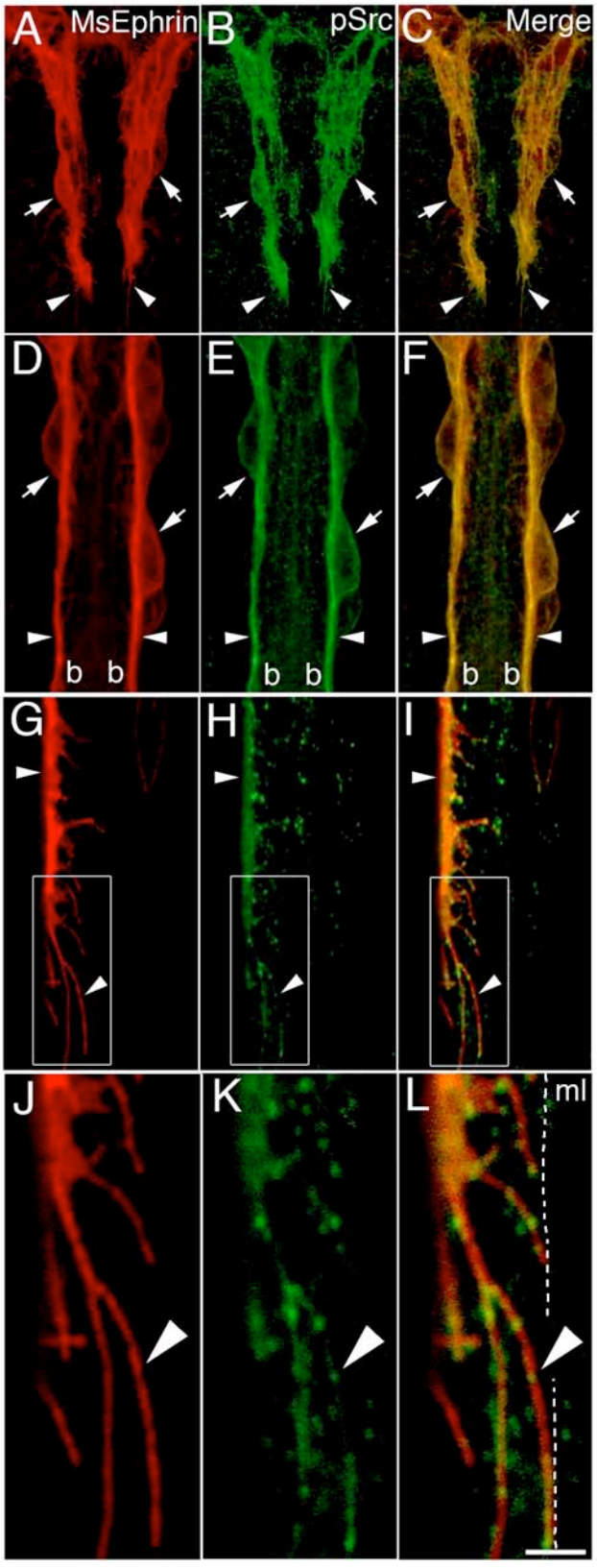


Figure 3.

Figure 3. Phosphorylated Src proteins are localized within the EP cells and their leading processes as they migrate on the migut muscle bands. *A-C*: whole-mount immunostaining of the EP cells in an embryo at 58% of development (near the onset of migration) using antibodies against MsEphrin (red) and phosphorylated Src (green). The merged view in panel C shows significant colocalization between MsEphrin and pSrc. The arrows point to the soma of the EP cells and the arrowheads point to the leading filopodial arrays. *D-F*: a similarly immunostained preparation at 65% of development. MsEphrin and pSrc continue to colocalize in the EP cells' somata (arrows) and in the fasciculated bundles of axons that are projecting posteriorly (arrowheads). pSrc is also detectable at low levels in the muscle band pathways (b). *G-I*: MsEphrin and pSrc both localize to the distal portion of the leading axons (arrowheads) where filopodia explore the migratory environment. The growth cone on the right side of the midline is not visible in this micrograph. *J-K*: a high magnification view (from the boxed region in G-I) shows that MsEphrin and pSrc colocalize in filopodia that approach, but do not cross the midline. The dashed line in L delineates the muscle band pathway from the midline (ml). In these 2 μM confocal Z-stacks, pSrc appears more punctate (see arrowheads) suggesting it may be preferentially localized into discrete microdomains. Scale bar = 4 μm in J-L; 12 μm in A-I.

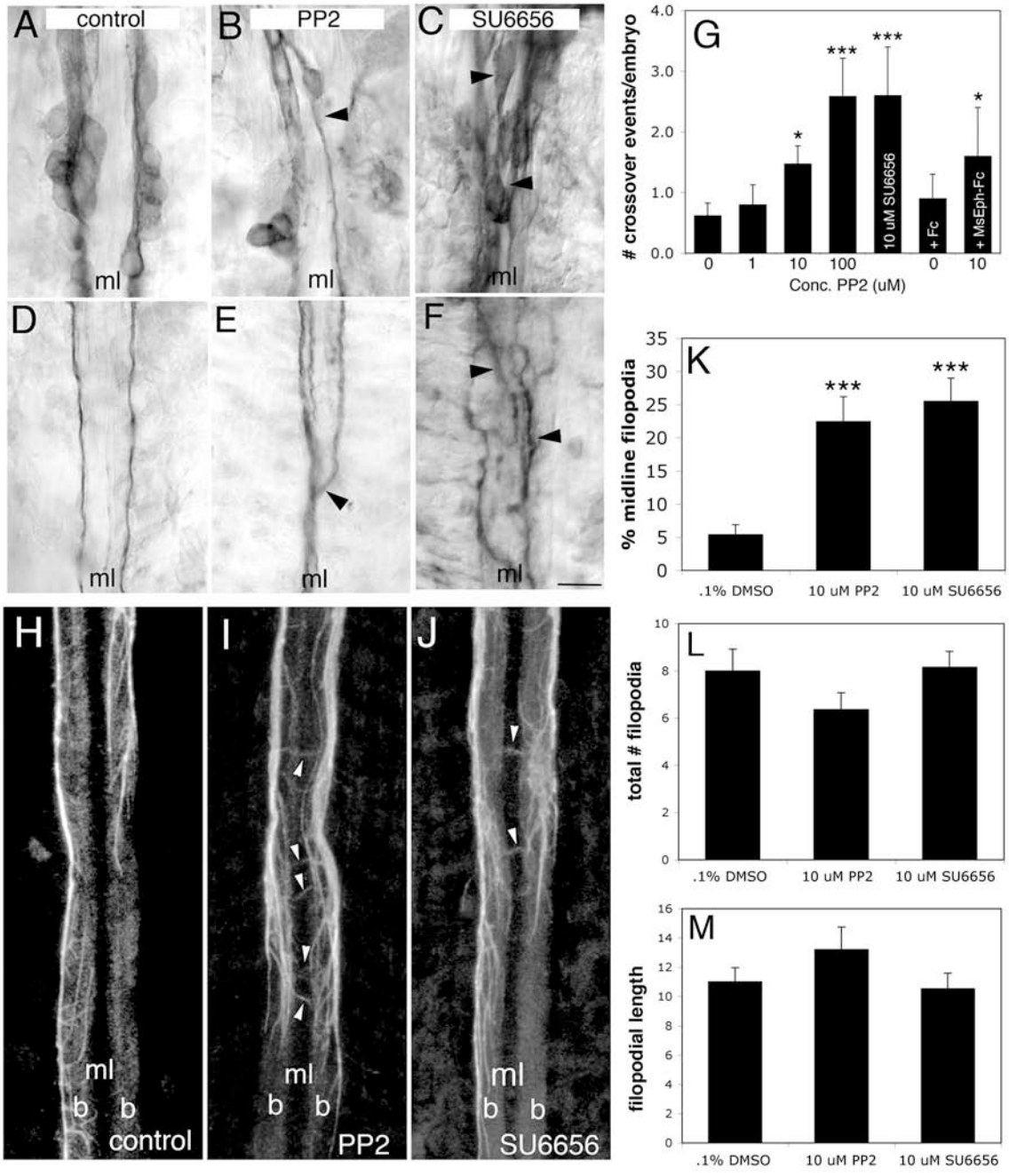
arrowheads) and growth cones as they traversed posteriorly along the midgut muscle bands (Fig. 3G-I; arrowheads). By comparison, only faint levels of pSrc expression could be detected in the underlying muscle bands and surrounding gut musculature, which do not express MsEphrin (Coate et al., 2007). At higher magnification (Fig. 3J-L), MsEphrin antibodies uniformly labeled the leading processes and filopodia extended by the EP cells along the bands (as previously reported) (Coate et al., 2008), whereas anti-pSrc antibodies produced a more punctate pattern of staining in the growth cone filopodia, as well as more sparsely in the underlying muscle cells. Possibly, this punctate pattern of staining reflects the preferential distribution of pSrc within membrane microdomains in other cell types (Stefanova et al., 1991), although the nature and possible function of these domains has not yet been characterized within the developing nervous system. The co-localization of MsEphrin with activated Src the most motile

regions of the migratory EP cells and their processes suggests that they may functionally interact in response to MsEph-receptors encountered at the midline.

Inhibiting MsSrc in the migrating EP cells causes midline crossover phenotypes.

Previously, we showed that blocking endogenous MsEphrin-MsEph receptor interactions in the developing ENS or knocking down MsEphrin expression in the EP cells led to aberrant midline crossing by the neurons and their growing processes, consistent with the model that reverse signaling via MsEphrin regulates this aspect of EP cell behavior (Coate et al., 2008). If Src-related kinases are essential to the transduction of MsEphrin reverse signaling (as has been suggested for Ephrin-A reverse signaling *in vitro*), then we would predict that inhibiting Src activation should generate similar crossover phenotypes. When embryos were cultured overnight in the presence of 0.1% DMSO (as a control), the migratory neurons maintained their normal course along the band pathways, never crossing the midline (**ml**; Fig. 4A). By contrast, when treated with the Src inhibitors PP2 (Fig. 4B) or SU6656, the migratory EP exhibited a marked increase in midline crossovers (Fig. 4B-C, arrowheads), similar to their behavior following disruption of MsEphrin-MsEph-dependent interactions (Coate et al., 2008). Similarly, both Src inhibitors also caused a dramatic increase in midline crossovers by their leading processes and growing axons (Fig. 4E-F), contrary to the precise trajectories along the band pathways that they normally maintain (Fig 4D). When we quantified the frequencies of midline crossing events in the different treatment groups, we found that PP2 induced midline crossing in a dose-dependent manner (from 1-100 μM) that was statistically different from controls at concentrations of 10 μM or higher (Fig. 4G).

Likewise, treatment with 10 μM SU6656 also caused a highly significant increase in crossover events (Fig. 4G). Notably, however, these treatments did not lead to other guidance defects: the EP cells did not migrate inappropriately onto the lateral interband musculature, nor was their overall extent of migration and outgrowth on the muscle bands perturbed (described below). Thus, inhibiting endogenous MsSrc activity in the EP cells resulted in the same 'gain-of-function' phenotype that we observed when we blocked MsEphrin-dependent reverse signaling, whereby the neurons migrated and extended processes inappropriately onto the midline cells. As the EP cells migrate, they rapidly extend fine filopodia onto both the band pathways and adjacent interband regions. Those filopodia that contact the muscle bands tend to be incorporated into their leading processes, while filopodia that contact the midline cells (and lateral interband regions) are usually rapidly retracted (Copenhaver, 2007). Previously, we developed an assay for quantifying the filopodial behavior of the EP cells in short-term culture: blocking MsEph receptors on the midline cells caused a significant increase in the number of filopodia that grew onto the midline (Coate et al., 2008). In the context of our other experiments, this assay provided further evidence that the MsEph receptors induce filopodial retraction at the midline by stimulating MsEphrin-dependent reverse signaling in the neurons. When we treated the migrating EP cells with PP2 and SU6656 in this assay, we observed a marked increase in the number of filopodia that extended onto the midline cells (Fig. 4I-J, arrowheads) in contrast to control preparations, in which nearly all filopodia remained on the band pathways (Fig. 4H). As shown in Fig. 4K, this increase in ectopic filopodia on the midline cells was highly significant for both Src inhibitors. Interestingly, the Src inhibitors caused no differences in either the number of filopodia per growth cone (Fig


A
control
B
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crossover events/embryo
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PP2
SU6656
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filopodial length
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14
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2
0
.1% DMSO
10 uM PP2
10 uM SU6656

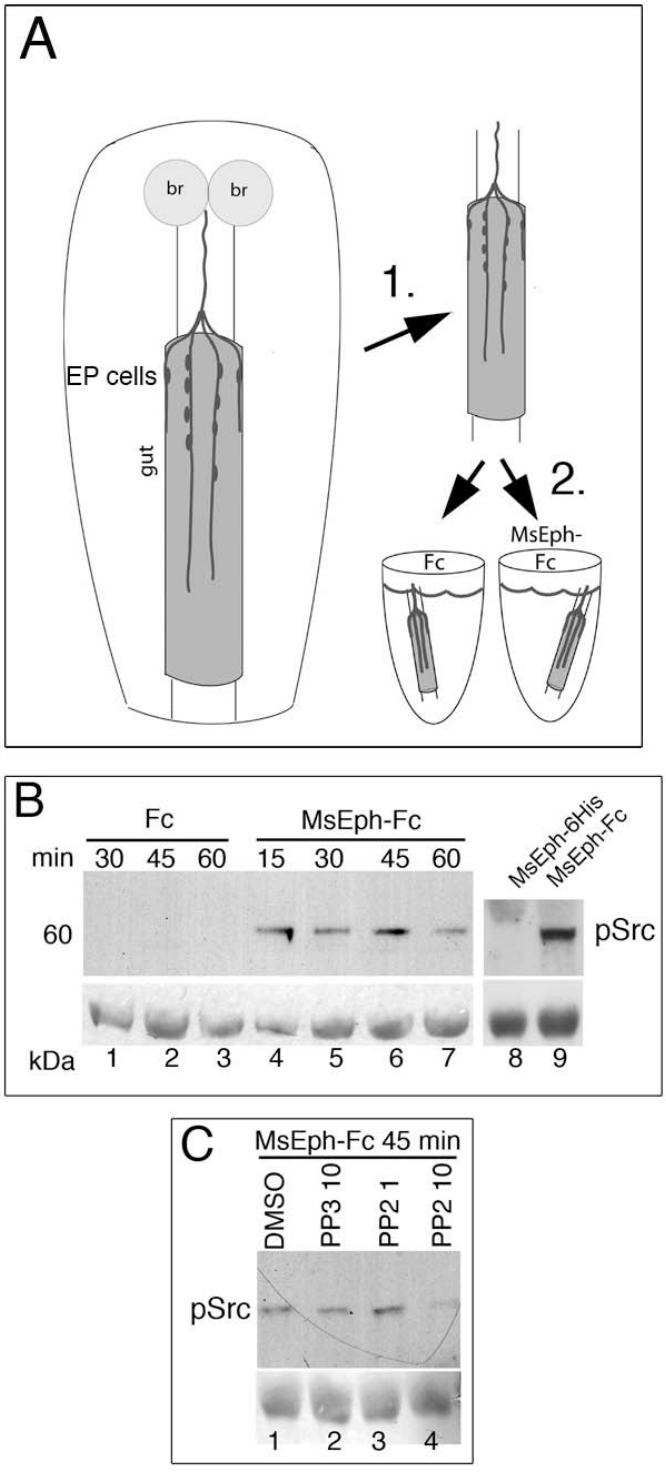
Figure 4.

Figure 4. Manipulations of Src activity *in vivo* lead to midline crossing phenotypes. *A-F*: pharmacological inhibition of Src in overnight embryo culture assays. Post-culture embryos were immunostained with anti-Fas II antibodies. *A*: an embryo treated with control culture medium + DMSO shows normal migration along the band pathways. *B*: treating embryos with PP2, which is known to inhibit tyrosine phosphorylation within the activation loop of Src, leads to midline crossing by migratory neurons. *C*: likewise, midline crossing by migratory neurons was observed after treating embryos with SU6656, which inhibits Src kinase activity. *D*: the leading processes of control embryos showed no midline crossing. *E* and *F*: severe midline crossing by leading processes was observed after treating embryos with Src inhibitors. In A-F, the arrowheads point to cells or processes that have crossed the midline. ml = midline. *G*: histogram illustrating the number of cross-over events per embryo among different treatment groups. PP2 induces midline crossing in a dose-dependent manner. SU6656 at 10 μM also leads to midline crossing. The addition of control Fc had no change in midline crossing frequency compared to controls. The addition of 20 μg/ml MsEph-Fc to 10 μM PP2 did not significantly change the rate of midline crossing compared to 10 μM PP2 alone. *H-J*: Src inhibitors also cause motile filopodia to aberrantly stray over the midline in short-term 3-hour cultures. Post-culture embryos were immunostained with anti-Fas II antibodies. *H*: control embryos exhibit little-to-no midline crossing. *I* and *J*: both Src inhibitors cause a high percentage of filopodia to invade the midline. The arrowheads point to filopodia that have aberrantly crossed the midline. ml = midline; b = muscle band pathway. *K*: histogram showing the average percent of filopodia among treatment groups that crossed the midline in short-term assays (H-J). *L*: histogram comparing the average number of filopodia per growth cone among treatment groups. No differences were observed. *M*: histogram comparing the average filopodial length among treatment groups. No differences were observed. Statistics: Students 2-tailed t-test; *$p \leq 0.05$; ***$p \leq 0.001$. Scale bar in F = 12 μm for A-F; 5 μm for H-J.

4L) or the average length of individual filopodia (Fig. 4M), compared to control groups. These results suggest that within the developing ENS, the primary role of MsSrc-dependent signaling in migrating neurons may be to modulate filopodial dynamics in response to local guidance cues (including MsEph receptors at the midline), rather than regulating more general aspects of growth and motility, as has been proposed from experiments performed *in vitro* (Robles et al., 2005).

Reverse signaling by MsEphrin activates MsSrc in the ENS.

We previously demonstrated that overstimulating reverse signaling in the *Manduca* ENS (using dimeric MsEph-Fc fusion proteins) inhibited neuronal migration


A
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60
pSrc
kDa 1 2 3 4 5 6 7 8 9
C
MsEph-Fc 45 min
DMSO
PP3 10
PP2 1
PP2 10
pSrc
1 2 3 4

Figure 5.

Figure 5. MsEphrin-mediated reverse signaling activates Src in isolated guts. *A*: Schematic representation of the Gut isolation assay. (Step 1) the entirety of the gut is removed from the body cavity. (Step 2) individual guts are placed in microcentrifuge tubes containing either control Fc or MsEph-Fc at 25 μg/ml and allowed to incubate for different times. Previous studies have shown that MsEph-Fc only identifies MsEphrin expressed by the EP cells. *B*: a Western blot probed with anti-pSrc antibodies. Each lane represents the lysate from 1 individual gut. Nitrocellulose membranes stained with Ponceau S (lower panels) show equivalent loading (the protein identity is unknown). (Lanes 1-3) treatment with Fc alone led to no phosphorylation of Src. (Lanes 4-7) MsEph-Fc treatment, however, led to Src phosphorylation by 15 minutes, with the intensity of the signal beginning to taper by 60 minutes. (Lanes 8 and 9) monomeric MsEph-6His, which is not competent to induce reverse signaling, fails to activate Src unlike dimeric MsEph-Fc (45 minute treatment). kDa = kilodaltons; min = minutes. *C*: (lanes 1-3) a 20 minute pre-treatment of DMSO alone, PP3 or PP2 at 1 μM has no effect on Src phosphorylation in the presence of MsEph-Fc. (Lane 4) 10 μM of PP2, however, significantly decreases the ability of Src to become phosphorylated (a negative control).

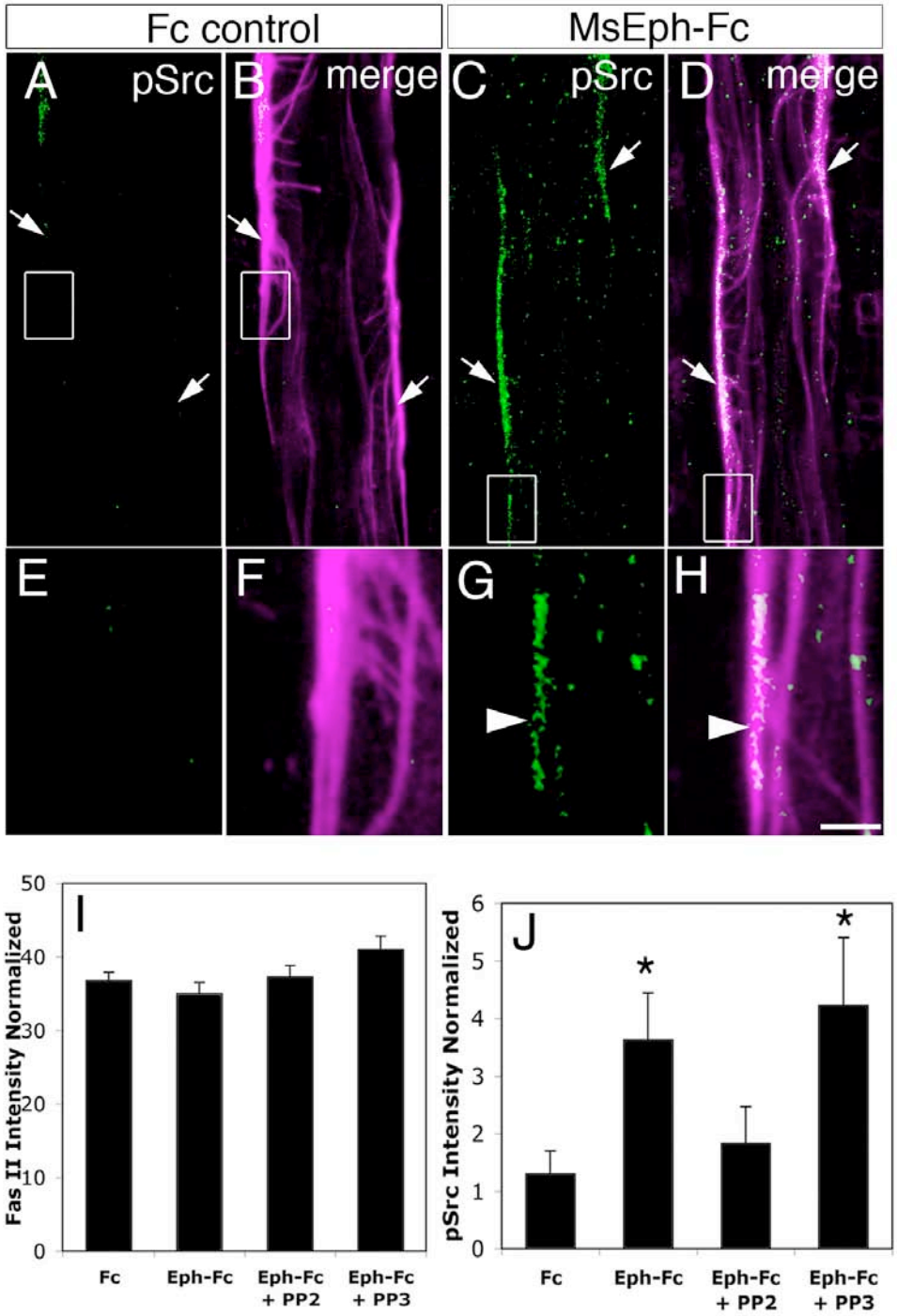
and outgrowth, supporting the model that endogenous MsEph receptors at the enteric midline normally repel the EP cells by activating this signaling pathway (Coate et al., 2008). In transfected fibroblasts, reverse signaling via type-A Ephrins was shown to potentiate the phosphorylation of several phosphoproteins, including candidate SFKs (Davy et al., 1999; Huai and Drescher, 2001). To test whether reverse signaling by MsEphrin similarly promotes Src phosphorylation, we established an explant assay whereby the phosphorylation of MsSrc in the developing ENS could be examined biochemically (Fig. 5). Individual guts were dissected from embryos at 60-62% of development (Fig. 5A step 1) and transferred into microcentrifuge tubes containing either MsEph-Fc or control Fc fusion proteins (step 2). Because our previous studies showed that MsEph-Fc only bound the MsEphrin-positive EP cells (Coate et al., 2007), we predicted that this protocol should allow us to test the effects of these proteins in an EP cell-specific manner. After incubation at room temperature for varying intervals, protein extracts from the individual guts were probed in Western blots with anti-pSrc (Y416)

antibodies (Fig. 5B-C). As shown in Fig. 5B (lanes 1-3), treating isolated guts with Fc control proteins induced no detectable increase in pSrc levels over the time course of this experiment (15-60 min). In contrast, exposure to MsEph-Fc fusion proteins led to a dramatic increase in MsSrc phosphorylation (Fig. 5B, lanes 4-7) with maximal levels achieved by 15 minutes and a gradual diminution in pSrc levels by 60 minutes. The concentration of MsEph-Fc used in these studies (25 μg/ml) was previously shown to induce an intermediate phenotype in cultured embryos, indicating that it only partially activated MsEphrin reverse signaling in the EP cells.

We also previously showed that while MsEph-Fc constructs (which spontaneously dimerize) stimulate MsEphrin reverse signaling in cultured embryos, MsEph-6His fusion proteins (which do not dimerize) block endogenous MsEphrin-MsEph interactions without inducing reverse signaling within the EP cells (Coate et al., 2008). We therefore treated individual guts with either MsEph-6His or MsEph-Fc proteins for 45 min and compared the resultant levels of MsSrc phosphorylation. As shown in Fig. 5B (lanes 8-9), MsEph-6His proteins induced no detectable increase in pSrc levels, compared to the robust effect of MsEph-Fc; this result supports our previous conclusion that simply occupying MsEphrin ligands with monomeric receptor constructs is not sufficient to induce MsEphrin-dependent reverse signaling in the EP cells. To confirm the specificity of this result, we pre-treated gut explants with PP2, which is known to inhibit the phosphorylation of Src kinases within their activation loops (Hanke et al., 1996). As a control, we also pre-treated a matched set of preparations with PP3, a structurally related molecule that does not affect SFK activation. After 25 minutes, MsEph-Fc was added to the incubation medium (to a final concentration of 25 μg/ml) for

another 45 min. As shown in Figure 5C, pre-treating explants with either 0.1% DMSO (lane 1) (as a vehicle control) or the inactive analogue PP3 (10 μM; lane 2) had no effect on the ability of MsEph-Fc to subsequently activate MsSrc. Likewise, pre-treatment with 1 μM PP2 (a concentration that also did not induce ectopic midline crossovers in embryo culture) did not inhibit Src activation by MsEph-Fc (Fig. 5C, lane 3). However, pre-treatment with 10 μM PP2 markedly reduced the ability of MsEph-Fc to activate MsSrc (lane 4); this same concentration caused a significant increase in the number of crossover phenotypes by the EP cells *in vivo* (Fig. 4). These results indicate that activating MsEphrin-dependent reverse signaling stimulates MsSrc phosphorylation in the ENS, consistent with the hypothesis that Src kinases play an important role in this process.

Next, to examine whether stimulation of MsEphrin on the EP cells leads to a stimulation of Src activity specifically in these neurons, we treated the developing ENS in cultured embryos (at 60-62% of development) with either MsEph-Fc or control Fc proteins (25 μg/ml). In addition, we also pre-treated matched preparations with either PP2 or PP3, prior to the addition of MsEph-Fc. We then rapidly fixed the preparations and double-immunostained them with anti-MsFas II (as a neuronal marker) and anti-pSrc. The growth cones and associated filopodia of the EP cells were then analyzed by confocal microscopy. In contrast to the preparations shown in Figure 3, for these experiments, we used the anti-pSrc antibody at a dilution of 1:500 rather than 1:00, which in pilot studies allowed us to measure pSrc immunofluorescence within a linear range. At this dilution, pSrc staining was barely detectable in the EP cells above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Fas II Intensity Normalized
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Eph-Fc + PP2
Eph-Fc + PP3

Figure 6.

Figure 6. MsEphrin-mediated reverse signaling activates Src in growth cones *in vivo*. *A-H*: embryos were treated for 25 minutes with 25 μg/ml Fc or MsEph-Fc then probed with dilute concentrations of anti-pSrc antibodies (green), and then counterstained with MsFas II antibodies (magenta) to mark the leading processes. *A-B*: Fc treated preparations show very little if any pSrc present in the leading growth cones. *C-D*: MsEph-Fc, in many preparations, led to a robust enrichment of pSrc in the leading growth cones, showing that MsEphrin-mediated reverse signaling activates Src. The arrows in A-D point to the growth cones. *E-H*: high-magnification views (from the boxed regions in A-D) show the leading filopodial processes. *E-F*: Fc treatment shows little pSrc staining. *G-H*: in many cases, high levels of pSrc was observed in the leading filopodia of MsEph-Fc-treated growth cones. *I*: histogram showing that, among all treatment groups, MsFas II levels were unchanged (as an internal control). *J*: histogram showing that MsEph-Fc led to nearly a three-fold change in pSrc fluorescence in the leading filopodia. Pre-treating embryos with 10 μM PP2 prevented Src phosphorylation; 10 μM PP3 had no effect. Statistics: Students 2-tailed t-test; *$p \leq 0.05$. Scale bar in H = 10 μM for A-D; 2.5 μM for E-H.

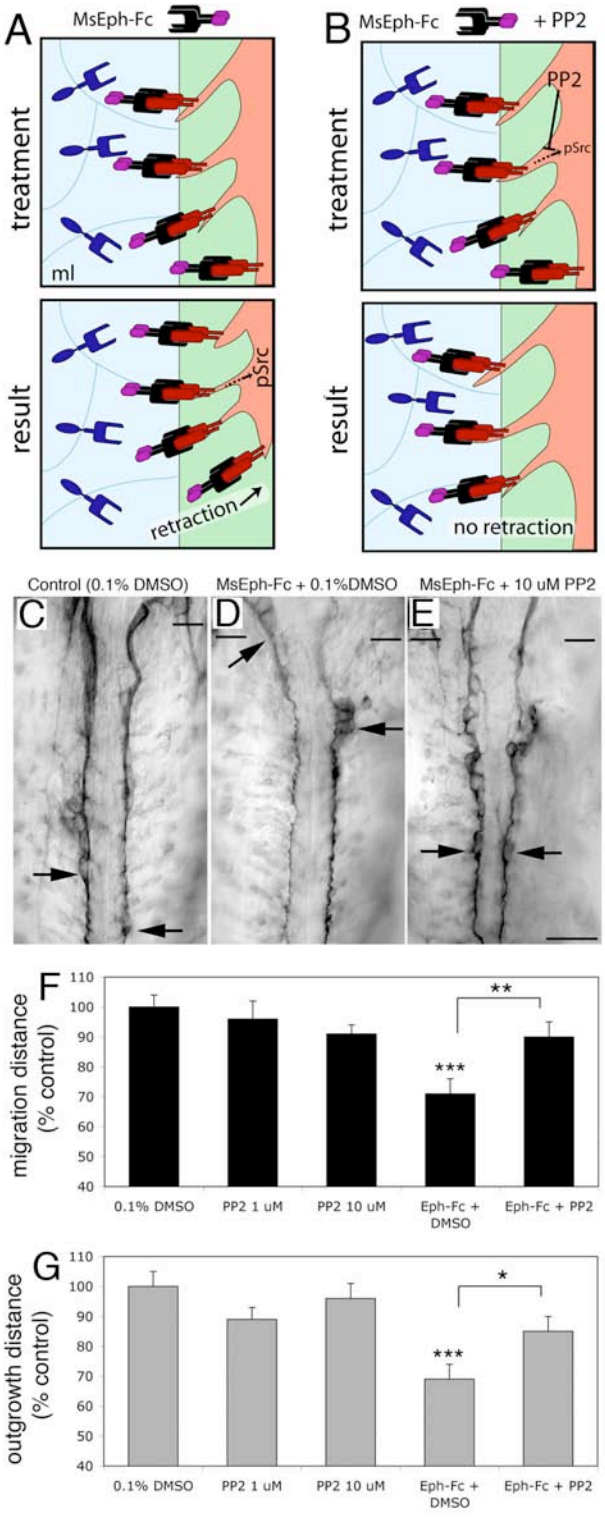
background fluorescence. For each preparation, we captured a 3.5 μm confocal Z-stack of the leading filopodia (8 X 0.5 μm sections). As shown in Figure 6A-B, the growth cones of EP cells treated with Fc alone exhibited only low levels of pSrc immunoreactivity (green), whereas growth cones treated with MsEph-Fc (to stimulate MsEphrin reverse signaling) exhibited substantially stronger levels of pSrc staining, well above background fluorescence (Fig. 6C-D). The resolution of the EP cell processes provided by MsFas II counterstaining also allowed us to visualize pSrc levels within filopodia extending in advance of the growth cones (Fig. 6E-H). In preparations treated with Fc alone (Fig. 6E-F), only low levels of pSrc staining could be detected within the filopodia (Fig. 6F). In contrast, much higher levels of pSrc were seen in the filopodia of EP cells treated with MsEph-Fc (Fig. 6G-H). To quantify this effect, we used ImageJ software to measure the relative intensities of pSrc immunoreactivity within the filopodia of these preparations (normalized to background levels in adjacent muscle cells lacking MsEphrin); concurrently, we also measured the relative levels of MsFas II

immunofluorescence in the same regions to control for volumetric artifacts (following our published methods) (Coate et al., 2008). For this quantification, the two most superficial confocal sections of each preparation (2 X 0.5 μm) were extracted and flattened. As shown in Figure 6I, no significant variations were observed in relative MsFas II immunofluorescence among any of the treatment groups. In contrast, relative levels of pSrc immunoreactivity were almost 300% higher in preparations treated with MsEph-Fc, compared to preparations treated with control Fc proteins (Fig. 6J). Notably, pre-treatment with PP2 almost completely abolished this response, whereas pre-treatment with PP3 had no effect, consistent with the results obtained in our gut explant assay (Fig. 5). These results indicate that overstimulating MsEphrin reverse signaling in the EP cells results in a highly significant increase in MsSrc activation, an effect that can be detected within the leading processes and exploratory filopodia extended by the migrating neurons within the developing ENS.

MsSrc is necessary for MsEphrin-dependent reverse signaling in the migratory EP cells.

In previous studies performed in embryo culture, we used MsEph-Fc fusion proteins to hyperactivate MsEphrin-dependent reverse signaling in the EP cells, which resulted in a potent inhibition of their migration and outgrowth (Coate et al., 2008). Furthermore, the results shown in Figures 5-6 indicated that the stimulation of MsEphrin reverse signaling resulted in MsSrc phosphorylation within the migratory neurons and their leading processes, but they did not address whether this mechanism was necessary for ENS development. As shown schematically in Figure 7A, we hypothesized that when

MsEph-Fc dimers (black) bind to MsEphrin (red) on the filopodia of migrating EP cells (upper panel), they overstimulate the response normally induced by endogenous MsEph receptors on the midline cells (blue). This hyperactivates MsSrc in the leading processes of the neurons, causing retraction of their filopodia, and a global inhibition of their motile behavior (Fig. 7A, lower panel). If Src activation is necessary for this response to MsEph-Fc, then the inactivation of MsSrc with PP2 should prevent this response (Fig. 7B, upper panel), allowing the neurons to migrate normally (Fig. 7B, lower panel). We therefore used our embryo culture preparation to test whether the effects of MsEph-Fc treatments on EP cell motility in vivo requires MsSrc. In embryos treated with 0.1% DMSO alone, the EP cells migrated normally, remaining closely associated with their band pathways while avoiding the inhibitory midline domains (Fig. 7C). Treating the EP cells at the onset of their migration (~55% of development) with an intermediate concentration of MsEph-Fc caused a significant decrease in the extent of their migration and axon outgrowth (Fig. 7D), as previously reported (Coate et al., 2008). At 20 μg/ml, MsEph-Fc treatments caused a 25% decrease in the distance of migration and a 30% decrease in axon outgrowth, respectively (Fig. 7F-G). However, when we treated the developing ENS with 10 μM PP2 plus MsEph-Fc, the extent of EP cells migration and outgrowth were almost fully restored (Fig. 7E; 7F-G). In addition, MsEph-Fc did not alter the extent to which PP2 induced midline crossing (Fig. 4G), suggesting that MsEph-Fc simply blocked MsEphrin-MsEph complexes when it was unable to promote reverse signaling via Src. These results indicate that MsSrc activation within the EP cells is indeed necessary for the control of their behavior by MsEphrin-mediated reverse signaling.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0.1% DMSO
PP2 1 uM
PP2 10 uM
Eph-Fc + DMSO
Eph-Fc + PP2

Figure 7.

Figure 7. Src phosphorylation is necessary for MsEphrin-mediated reverse signaling in vivo. *A*: schematic illustration of the effects of MsEph-Fc on EP cell migration. Exogenous MsEph-Fc (black) binds MsEphrin (red) expressed by the EP cells, mimicking the normal activity of midline MsEph receptors (blue; ml). Reverse signals are predicted to phosphorylate Src, inducing filopodial retraction. *B*: similar scenario as in A, but the addition of PP2 blocks Src phosphorylation via reverse signaling, permitting normal migration distance when the neurons are confronted with MsEph-Fc. *C-E*: embryo culture preparations immunstained with MsFas II antibodies. The black bars denote the foregut-midgut boundary. The black arrows point to the EP cell that migrated the furthest. *C*: an embryo treated with control culture medium + DMSO exhibits normal migration. *D*: as shown previously, MsEph-Fc treatment inhibits migration by over-stimulating MsEphrin-dependent reverse signaling. *E*: a preparation treated with both PP2 and MsEph-Fc shows almost normal migration. *F*: histogram illustrating EP cell migration distances among different treatment groups as a percent of control. *G*: histogram illustrating axon outgrowth distances among different treatment groups as a percent of control. Scale bar in E = 20 μm. Statistical analysis: Students 2-tailed t-test; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

DISCUSSION

We have identified a *Manduca* Src family kinase (SFK) as key component of MsEphrin-mediated reverse signaling during ENS development. In previous work, we proposed a model whereby *reverse* and not *forward* signals resulting from MsEph-MsEphrin interactions functioned to steer the migratory EP cells away from the midline (Fig.1; Coate et al., 2008). Our model now predicts that midline MsEph receptors normally activate neuronally-expressed MsEphrin, which in-turn leads to the phosphorylation of Src as a necessary component of midline repulsion. This report represents one of the first examples of a specific mechanism by which a GPI-linked Ephrin transmits a reverse signal to modulate a specific cellular behavior *in vivo*.

Although we isolated both *Manduca* Src homologues (MsSrc42 and MsSrc64; Fig.2), we were not able to distinguish which one may be associated with cell-cell repulsion in this system, as both were expressed at relatively equivalent levels in the embryo. In future studies, it will be important to understand if there are any mechanistic differences between MsSrc42 and MsSrc64, and if there are any correlations with similar functions of Src in *Drosophila*. For example, both *Drosophila* Src64 and Src42 were shown to negatively regulate cell adhesion during trachea morphogenesis by regulating the turnover rate of E-cadherin and the activity of armadillo (Shindo et al., 2008). Intriguingly, these authors observed a strong enrichment of active Src42 (pSrc) in cells undergoing mesenchymal rearrangements, although all cells appear to express Src itself (Shindo et al., 2008). We observed a similar relationship of inactive and active Src on the outer surface of the developing gut: nearly all cells were stained positively with our non-phospho-Src antibody (Fig. 2E), but pSrc appeared to be concentrated mainly in the

EP cells during all phases of motility (Fig. 3). In this report, we also argue that Src downregulates cell adhesion by promoting filopodial retraction, but it is doubtful that adherens junctions are involved; when treated with PP2 or SU6656, the EP cells were only misdirected across the midline, but never assumed a more epithelial-like state (Fig. 4). However, we cannot rule out the possibility that Src may be involved with adherens junctions that may transiently form between the EP cells and the midline cells, or between two migrating EP cells. In future studies, it will be important to examine possible roles of E-cadherin/armadillo with respect to MsEphrin-MsEph interactions and Src activity.

In this report, we observed strong colocalization of pSrc and MsEphrin (Fig. 3), in accordance with previous reports where Ephrin-A ligands have been reported to colocalize with cytoplasmic phosphoproteins (Davy et al., 1999; Huai and Drescher, 2001). These studies, however, made the additional observation that these molecules were partitioned within membrane microdomains, a typical behavior of GPI-linked ligands and myristylated proteins such as Src (Chen et al., 2006; Mayor and Riezman, 2004). Does MsEphrin activate Src within a membrane microdomain? The 2 μM confocal Z-stacks revealed that the appearance of pSrc at the membrane was punctate, particularly in the filopodia of the leading proceses (Fig. 3; see K and L), but it is unknown in this system what (visually or biochemically) constitutes a lipid raft. Future investigations using known biochemical techniques to isolate microdomains or "detergent insoluble glycolipid enriched complexes" (DIGS)(Anderson, 1998) from the EP cells will help to understand whether Ephrin-As and Src family kinases associate within these complexes *in vivo*.

In previous studies, disrupting normal MsEph-MsEphrin interactions at the midline in long-term embryo culture assays (using exogenous fusion proteins or antisense Morpholinos) led to aberrant midline crossing of the EP cells during migration and outgrowth. Additionally, in short-term assays, blocking midline MsEph receptors (using MsEphrin-Fc) caused motile filopodia to ectopically invade the midline (Coate et al., 2008). In the present study, we observed similar midline crossover phenotypes when we inhibited Src activity by adding PP2 and SU6656 to cultured embryos in long- and short-term experiments (Fig. 4). Intriguingly, the phenotypes resulting after inhibiting Src were highly specific, appearing to be limited to misdirecting the neuronal processes over the midline; surprisingly we did not detect any changes in migration or outgrowth distance (Fig. 7F-G), or changes in filopodial number or length (Fig. 4L-M). Previously, studies *in vitro* have shown that Src kinases are associated with microtubule stability at the leading edge (Suter et al., 2004), and may promote filopodial extension (Robles et al., 2005), therefore it is surprising that the length of the filopodia on the EP cells' growth cones was not altered by the addition of PP2 or SU6656. One possible explanation for this difference is that additional guidance cues on the muscle bands, such as Fasciclin II (reviewed by Copenhaver, 2007), may help stabilize the filopodia, preventing them from either overextending or collapsing. An additional possibility is that Src may have different roles and interact with different effectors depending on the neuronal subtype and model system used.

Ephrin B reverse signaling has been widely described *in vivo* and many of the downstream signaling mechanisms have been elucidated (Kullander and Klein, 2002; Pasquale, 2005; Wilkinson, 2001). Contact with EphB receptors leads to phosphorylation

within the C-terminus of Ephrin-Bs (Bruckner et al., 1997; Holland et al., 1996; Kalo et al., 2001) by Src family kinases (Palmer et al., 2002), which then recruits SH2/SH3 domain-containing proteins to promote downstream signaling (Cowan and Henkemeyer, 2001). Alternatively, PDZ-adaptor proteins may be recruited to a receptor-bound Ephrin-B ligand (Bruckner et al., 1999) to regulate heterotrimeric G-protein signaling (Lu et al., 2001). In contrast, very little is known about the signaling mechanisms associated with Ephrin-A reverse signaling. Here, we demonstrated that hyperstimulating MsEphrin reverse signaling using MsEph-Fc fusion proteins *in vivo* increased the phosphorylation state of Src. This increase was observed biochemically using isolated guts (Fig. 5) and visually within the leading growth cones of the EP cells (Fig. 6). These results are reminiscent of findings from *in vitro* studies by Davy et al., where Fyn became increasingly phosphorylated in response to Ephrin-A5 activation (Davy et al., 1999). In *Manduca* there is no Fyn per se, but it is possible that one (or both) of the prototypical Src kinases and the vertebrate Fyn behave similarly in this context. Interestingly, preliminary comparisons show that both MsSrc42 and MsSrc64 are most similar to Fyn out of all the vertebrate SFKs (not shown). It will be important, in subsequent whole-animal vertebrate studies, to determine the extent to which Fyn or other Src family kinases are necessary to mediate Ephrin-A reverse signaling. It will also be interesting to investigate similarities between Ephrin-A and Ephrin-B reverse signaling effectors that extend beyond Src, and the extent to which these similarities confer similar cellular functions such as repulsion, as shown here.

Interestingly, previous reports have suggested that Ephrin-A reverse signals favor an adhesion response, often via inside-out activation of the integrin pathway (Davy et al.,

1999; Davy and Robbins, 2000; Huai and Drescher, 2001; Knoll and Drescher, 2002). In these studies, the addition of soluble EphA receptors to cells expressing Ephrin-A5 led to an increase in the ability of cells to adhere to extracellular matrix in an integrin-dependent manner *in vitro*. In addition, it has been shown that adhesion of topographic projections within the vomeronasal system is mediated by what is likely Ephrin-A reverse signaling (Knoll and Drescher, 2002; Knoll et al., 2001a). This serves as one clear difference between past findings and those in this report: here, we argue that MsEphrin reverse signaling via Src favors repulsion from the midline, not adhesion. Previously, manipulating the ENS using MsEph-Fc in embryo culture inhibited EP cell migration and outgrowth, which we interpreted as the net outcome of overstimulating a repulsive response. An alternative interpretation is that MsEph-Fc treatment overstimulated an *adhesion* response, thereby preventing the cells from migrating because they were too tightly adhered to their muscle band pathways. If this model held true, one might expect that blocking MsEphrin reverse signaling in the EP cells would increase the distance of migration and outgrowth. In this report, however, we did not observe any noteworthy increases in the distance of migration or outgrowth when the EP cells were treated with PP2 or SU6656 (Fig. 7), or any increase in filopodial length or number (Fig. 4). While these results support a different role for Ephrin-A reverse signaling (repulsion), we cannot entirely rule out the possibility that Src dynamics also regulate adhesion complexes, as the EP cells do indeed express a variety of cell adhesion molecules including Fasciclin II (Copenhaver, 2007) and integrins (T.M.C. unpublished observations). Future investigations using this model system will identify players downstream of Src that mediate differential guidance responses.

How does a GPI-linked protein on the outside of a cell signal across the membrane to activate a cytoplasmic kinase? One reasonable prediction is that Ephrin-As employ a transmembrane coreceptor containing cytoplasmic phosphotyrosine residues to act as a recognition site for the SH2 domain of SFKs (Boggon and Eck, 2004; Roskoski, 2004), but no such factor has yet been identified. An alternative hypothesis is that the dimerization/clustering activity of GPI-linked Ephrins creates a microenvironment that highly favors the autoactivation of Src. In this model, a coreceptor is not needed. Indeed, GPI-anchored ligands are known to partition within lipid microdomains (Mayor and Riezman, 2004), as are the SFKs that contain the appropriate myristylation/palmitylation consensus sites (Resh, 1994). In addition, SFKs may dock to other proteins within a membrane microdomain via their SH3 site, in a phosphorylation-independent manner (Boggon and Eck, 2004). The receptor-mediated clustering of a GPI-anchored protein, such as MsEphrin may simultaneously cluster the constituents of the membrane microdomain, favoring changes in SFK conformation and autophosphorylation within the activation loop (Smart et al., 1981). Accordingly, we were only able to activate Src using the dimeric form of soluble receptor (MsEph-Fc), while the 6His-tagged version, which only blocks receptor-ligand binding (Coate et al., 2008), did not increase Src phosphorylation (Fig. 5).

The findings in this paper elicit multiple questions regarding the regulatory functions of Src during EP cell migration. For example, what downstream targets of Src facilitate filopodial retraction? One report on insulin secretion has shown that Ephrin-A-mediated reverse signaling may destabilize F-actin in a Rac-dependent manner (Konstantinova et al., 2007). In the *Manduca* ENS, several monomeric G-proteins are

expressed in patterns that overlap MsEphrin and pSrc (T.M.C. and P.F.C. unpublished observations), thus it is possible that reverse signaling modulates the actin cytoskeleton via one of these factors. A more complicated question relates to how the EP cells integrate both repulsive cues from the midline and adhesive cues on the muscle bands (Wright et al., 1999) in a balanced manner such that the net output is posterior migration. It is possible that Src acts as a convergence point between MsEphrin- and MsFas II-mediated intracellular signals, as cell adhesion molecules are also known to modulate Src dynamics in developing neurons (Kiryushko et al., 2006; Thelen et al., 2002). The *Manduca* ENS will provide a unique system to investigate these and other mechanisms in the context of neuronal migration *in vivo*.

CHAPTER 5

Conclusions and future directions

MsEphrin and MsEph are expressed in complementary domains during Manduca ENS formation.

In chapter 2 of this thesis, I demonstrated that MsEphrin and MsEph are expressed in distinct domains in the ENS: during periods of migration and outgrowth along the midgut, the EP cells express MsEphrin throughout their plasma membrane, including their leading processes and associated filopodia. In contrast, MsEph receptor expression is restricted to the enteric midline. For these studies, I used a battery of techniques to document the patterns of MsEphrin and MsEph expression, including *in situ* hybridization histochemistry, immunolocalization, and in vivo labeling (in cultured embryos) with MsEph- and MsEphrin-coupled Fc-fusion proteins. In particular, I observed that MsEphrin-positive filopodial processes protruding from the EP cells extended up to but never across the midline, where MsEph is expressed. I also provided additional evidence that only one Eph receptor and one Ephrin ligand is expressed in *Manduca* (and apparently in all other insects, as well), substantiating a previous report by Kaneko and Nighorn (2003). This finding is in contrast with the multiple isoforms of Ephrins and Eph receptors that are found in vertebrate systems, demonstrating this *in vivo* system provides a unique opportunity to investigate the developmental mechanisms of Ephrin-Eph interactions in the absence of functional redundancy. This work has been

published in the **Journal of Comparative Neurology** (Coate et al., 2007; volume 502, pp. 175-191).

A role for Ephrin-Eph receptor signaling during the formation of the ENS.

In chapter 3, I experimentally tested the hypothesis developed from the results of chapter 2, that MsEphrin-MsEph receptor interactions mediate cell-cell repulsion when the EP cells encounter (or interact with) the midline inhibitory region of the gut. I manipulated MsEphrin-MsEph receptor signaling within the developing ENS with Fc- and 6His-tagged fusion proteins, as well as using antisense morpholinos to attenuate the endogenous expression levels of MsEphrin in the migrating neurons. Using these approaches, I demonstrated that either blocking MsEphrin-MsEph receptor interactions or reducing MsEphrin expression levels led to midline crossing by the EP cells during their migration and axon outgrowth. In addition, I discovered that overstimulating MsEphrin-mediated reverse signaling (using MsEph-Fc fusion proteins) inhibited the overall distance that the EP cells migrated and extended their leading processes, a finding that led to a surprising development in our model. My results indicated that *reverse* signaling via MsEphrin (a GPI-linked ligand) and not *forward* signaling was the principal mechanism that governed repulsion between the EP cells and the midline (illustrated in Fig.5.1). This work has been published in the **Journal of Neuroscience** (Coate et al., 2008; volume 28, number 15, in press).

A Src family kinase mediates reverse signaling via MsEphrin

The foregoing investigations have generated a multitude of questions concerning the mechanisms by which MsEphrin-dependent reverse signaling leads to changes in neuronal motility and guidance. The primary issue that I addressed in chapter 4 concerned the signaling factors that are activated downstream of MsEphrin-mediated reverse signaling. In the course of these investigations, I discovered that the activated form of a *Manduca* Src kinase showed a high degree of colocalization with MsEphrin at the leading edge of the migratory EP cells. Pharmacological inhibition of Src activation *in vivo* led to midline crossover phenotypes that were similar to those observed after endogenous MsEphrin-MsEph receptor interactions were inhibited (described in chapter 3). Conversely, overstimulating MsEphrin-mediated reverse signaling increased the phosphorylation state of Src (illustrated in Fig.1). This effect was demonstrated both biochemically using midgut explants containing the ENS, and by immunohistochemical localization phospho-Src, using high-resolution confocal imaging of the leading growth cones. Most importantly, manipulations performed in embryo culture demonstrated that Src phosphorylation was necessary for MsEphrin-mediated reverse signaling during normal ENS development. These results provide the first *in vivo* demonstration that a Src family kinase becomes phosphorylated downstream of Ephrin-A reverse signaling in order to mediate cell-cell repulsion.

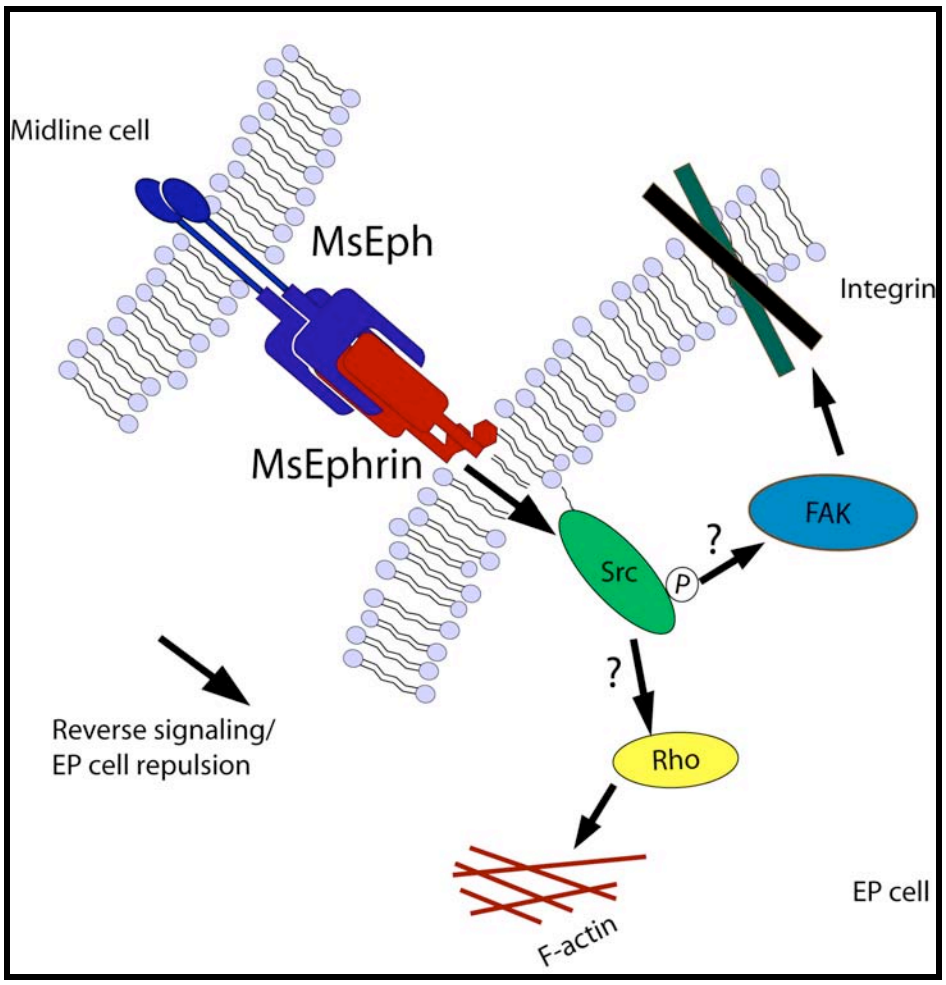

Figure 1. A model for MsEphrin-MsEph receptor signaling during ENS development in *Manduca*. Midline MsEph receptors contact MsEphrin expressed by the EP cells. This reverse signaling event leads to Src phosphorylation and EP cell repulsion from the midline. Future investigations will determine the extent to which other factors, such as Rho, focal adhesion kinase, and/or integrins, are required for MsEphrin-dependent EP cell guidance. These possible pathways (for future investigations) are delineated with a "?." Adapted from (Gauthier and Robbins, 2003; Konstantinova et al., 2007; Kullander and Klein, 2002)

FUTURE DIRECTIONS

This following overview describes several lines of investigation that could be pursued to advance the work described in my thesis. Although I have not provided an exhaustive list of experiments that should be performed for each study, I have included relatively straightforward experiments that might efficiently determine whether these lines of investigation will have merit.

Identification of the Src homolog that is downstream of MsEphrin reverse signaling.

We cloned both of the *Manduca* Src orthologues (MsSrc42A and MsSrc64B) and determined that both were expressed at relatively equal levels in the developing embryo (as judged by RT-PRC; Fig 4.2). Do the EP cells preferentially express either MsSrc42 or MsSrc64? We are currently generating MsSrc42- and MsSrc64-specific templates that will be used to synthesize antisense riboprobes for *in situ* hybridization histochemistry. RT-PCR may also be performed using an EP cell-specific cDNA library. Data in the Berkeley Drosophila Genome Project website (http://www.fruitfly.org/) indicates that both Src42 and Src64 are expressed ubiquitously throughout embryogenesis in fruitflies; thus it will be surprising if any differences are observed between the two orthologues in *Manduca*.

If the EP cells do express both MsSrc42 and MsSrc64, a more interesting question becomes whether there are differences in their subcellular distributions, and if either kinase co-localizes with MsEphrin during EP cell development. One report suggested that commercially available anti-pSrc (Y416) antibodies preferentially recognizes Src42 over Src64 in *Drosophila* (Shindo et al., 2008): overexpression of Src42 in the trachea led to

an increase in pSrc immunostaining, whereas the overexpression of Src64 did not. Using this strategy, MsSrc42 and MsSrc64 overexpression and subsequent pSrc immunostaining in tissue culture cells may allow us to distinguish which isoform is actually being detected (e.g. chapter 3, figure 3). In addition, Src42- and Src64-specific antibodies may be employed as another means of investigating their differential localization; antibodies against both factors are available (Shindo et al., 2008; Takahashi et al., 2005), although it is unknown whether they cross react with the *Manduca* orthologues.

As both MsSrc42 and MsSrc64 may be expressed in the EP cells, a function-based approach using antisense morpholino knock-down protocols may help identify which ortholog is activated downstream of MsEphrin. MsSrc42- or MsSrc64-specific morpholinos can be transfected into the developing ENS (as described in chapter 3, see chapter 3, Supp Fig. 3), followed by an analysis of midline crossing (using my published methods). If the depletion of either MsSrc42 or MsSrc64 levels results in midline crossover phenotypes, similar to the effects of PP2 or SU6656 treatment (chapter 4, Fig. 4), this result would provide strong evidence that one of these *Manduca* Src orthologues is required for MsEphrin-mediated reverse signaling. One pitfall to this approach is that morpholino knockdown of either Src may lead to phenotypes in other cells associated with the ENS besides the EP cells (including their underlying muscle band cells and the midline interband cells), as all cell types appear to express at least one form of Src (chapter 4, Fig. 2). Interestingly, RNAi-mediated knock down of Src42 in *Drosophila* only led to phenotypes in cells that expressed high levels of pSrc (Shindo et al., 2008). Likewise, my pharmacological treatments designed to inhibit Src within the developing

ENS in cultured embryos caused developmental defects that were specific to the EP cells (chapter 4, Fig. 4). Thus, it is plausible that Src morpholino treatment will also have EP-cell specific effects, providing clues as to the identity of the MsEphrin-dependent Src ortholog. However, the most effective means of testing whether manipulations of Src expression are specific to the EP cells would be to employ the methods for intracellular injection of single neurons in the developing ENS, as described in Horgan et al (1998).

Alternative means of overstimulating MsEphrin-mediated reverse signaling.

One obvious experiment missing from chapter 4 is an alternative means of overstimulating Src by methods other than MsEph-Fc treatment. Our model suggests that Src activation may be necessary for appropriate midline guidance via MsEph-MsEphrin interactions: PP2 and SU6656 treatments, which inhibited Src, led to crossing over in the presence of intact MsEph-MsEphrin complexes (chapter 4, Fig. 4). Conversely, I would predict that Src, in a constitutively active form, would completely inhibit the EP cells from migrating and projecting their axons, similar to MsEph-Fc treatments (chapter 4, Fig. 4-5). Constructs of either Manduca Src ortholog containing a point mutation replacing the key tyrosine residue within their repression loop with should generate mutant forms that are constitutively active (Y-F(500) in Ms-Src42A; Y-F(518) in Ms-Src64B; analogous to Y-F(527) in human c-Src)(Shi et al., 2006). The overexpression of a constitutively active Src was previously shown to modulate filopodial dynamics within cultured neurons (Robles et al., 2005), and thus may be a useful tool for manipulating the EP cells.

Possible downstream targets of reverse signaling I: the FAK-Integrin pathway

As illustrated in figure 1 (above), one possibility is that Src phosphorylation leads to the local recruitment of focal adhesion kinase (FAK) within the leading processes of the EP cells, which then may regulate integrin dynamics. As described in chapter 4, Ephrin-A reverse signaling has been shown to promote cell adhesion in cell culture via a signaling pathway that involves the phosphorylation of one or more cytoplasmic phosphoproteins, which in turn promote the activation of integrins ('inside-out signaling'; Davy et al., 1999; Davy and Robbins, 2000; Huai and Drescher, 2001). In neurons, a similar signaling cascade involving Src and FAK has been proposed to stabilize focal contacts mediated by the binding of integrins to extracellular matrix molecules (ECM), whereby FAK-dependent signaling promotes axonal outgrowth (Robles and Gomez, 2006). However, our current model argues that Src activation may play the opposite role, destabilizing MsEphrin-expressing filopodia that have made contact with midline MsEph receptors. It is possible, however, that the phosphorylation of Src in the EP cells leads to the recruitment of FAK at points of contact between the neurons and the underlying muscle bands, stimulating integrin-mediated adhesion to their appropriate pathways in lieu of the inhibitory midline cells (where MsEph receptors are expressed). By this model, there would have to be regional differences in the distributions of ECM molecules recognized by integrins on the EP cells, facilitating differential adhesion to the muscle bands rather than the midline cells (c.f. Geiger et al., 2001). Preliminary studies have shown that both FAK (not shown) and integrins (Fig. 2)

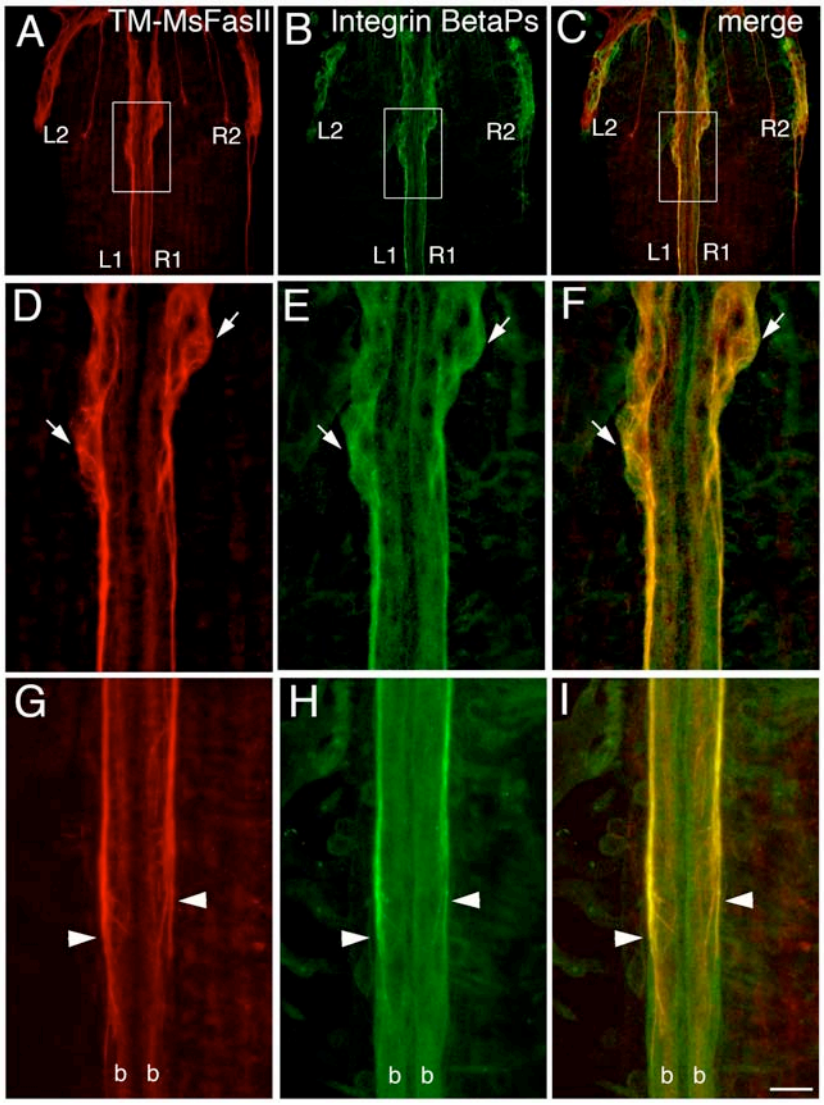

Figure 2. Integrins are expressed during EP cell development. *A-I*: An embryo at 65% of development was immunostained with antibodies against MsFas II (see chapters 2,3, and 4) and antibodies against *Drosophila* integrin βPs (1:100). *A-C*: A low-magnification image showing that integrin expression, like MsFas II, is prominent in the EP cells, although both are also expressed in the underlying muscle band pathways (b). *D-F*: A high-magnification image of the boxed region in A-C. MsFas II and integrin βPs are both associated with the plasma membrane of the migratory neurons (see arrows). *G-I*: Like MsFas II, integrin βPs also traffics into the most motile regions of the EP cells' leading processes (see arrowheads). Scale bar = 40 μm for A-C; 8 μm for D-I. In A-C, L = left; R = right.

are expressed in the EP cells during migration and outgrowth, although somewhat paradoxically, integrins are also expressed in the underlying muscle bands, as well.

Previously, studies of integrins in many systems (including *Drosophila*) have used RGD peptides to block the assembly of integrins with certain components of the ECM, including fibronectins (Bunch and Brower, 1992; Shi and Ethell, 2006). Based on these reports, we hypothesized that, if integrins are associated with MsEphrin-mediated reverse signaling in the EP cells, blocking their interactions using RGD peptides might result in ectopic midline crossing by the EP cells. However, preliminary experiments in which the developing ENS was treated with up to 50 μg/ml of RGD peptide generated no observable phenotype: the EP cells migrated normally without crossing the midline. It is possible, however, that the EP cells might be more sensitive to higher concentrations of RGD peptide; alternatively, integrins expressed by the EP cells might mediate adhesive interactions with ECM components other than the RGD motif. A more sophisticated method for manipulating integrin-dependent adhesion in the ENS would be to transfect the EP cells with a morpholino targeting the Manduca orthologues of the integrin subunits. These experiments would provide the foundation for future studies designed to test whether MsEphrin reverse signaling regulates neuronal migration and outgrowth in this system by modulating integrin-mediated cell adhesion.

Possible downstream targets of reverse signaling II: the Rho-GTPase pathway.

The Rho family of monomeric GTPases (Rac/Rho/Cdc42) modulate different aspects of actin assembly and disassembly and are well known for their effects on neuronal growth and guidance (Govek et al., 2005). Rac and Cdc42 are generally

thought to mediate neurite extension (stabilizing actin), whereas activated Rho is thought to mediate neurite retraction (destabilizing actin)(Govek et al., 2005), although there are important exceptions suggesting that their interactions in vivo may be substantially more complex (de Curtis, 2008; Linseman and Loucks, 2008). The Rho family of GTPases have also been proposed to act as effectors of forward and reverse signaling for both the A and B subclasses of Ephs and Ephrins (Govek et al., 2005; Konstantinova et al., 2007; Kullander and Klein, 2002). As illustrated in figure 1, the activity of Rho family GTPases can be regulated by SFKs (by virtue of their ability to regulate the RhoGAP family of activator proteins), and are thus good candidates for playing a role in the control of EP cell motility. The results of my research strongly suggests that reverse signaling via MsEphrin either mediates filopodial retraction or limits filopodial extension in the vicinity of the MsEph-expressing midline cells; thus, do Rho proteins participate in this response?

As shown in figure 3, my preliminary studies have also shown that the Rho1 immunoreactivity co-localizes with MsEphrin in the EP cells (using a monoclonal antibody against *Drosophila* Rho). The distribution of Rho1 and MsEphrin almost completely overlap: both can be detected in the somata of the EP cells (Fig. 3 D-F; see arrows) and in their exploratory filopodia extending from their leading growth cones (Fig. 3 G-I; see arrowheads).

There are several methods by which Rho might be manipulated within the developing ENS. First, embryos could be cultured in the presence of the C3 coenzyme, a Rho-specific inhibitor that has been shown to down-regulate neurite extension in a Rho-dependent manner (Hirose et al., 1998; Kamata et al., 1994; Nishiki et al., 1990), and

membrane-permeable forms of this inhibitor are now commercially available. If MsEphrin reverse signaling requires Rho activation to control EP cell behavior, then the C3 coenzyme would be predicted to generate midline crossover phenotypes, as observed after PP2 treatment (chapter 4, Fig. 4). Furthermore, if the GDP- and GTP-bound forms of Rho could be distinguished biochemically in small sample sizes, it would be possible to employ my midgut explant strategy to test whether stimulation of MsEphrin-mediated reverse signaling with MsEph-Fc (as described in chapter 4; Fig. 4.5) affects the activation state of Rho in the EP cells. Biochemical and *in vivo* experiments combining MsEph-Fc and C3 could then be performed to demonstrate whether the regulatory effects of MsEphrin reverse signaling on EP cell motility require Rho activation, and whether the effects of the inhibitor are specific to MsEphrin-dependent reverse signaling (or also impinge on other aspects of EP cell guidance).

In other preparations, the overexpression of constitutively activated Rho (CA-Rho) resulted in aberrant neurite retraction (Sebok et al., 1999), whereas dominant-negative forms of Rho (DN-Rho) have been shown to increase filopodia number in cultured *Xenopus* spinal neurons (Yuan et al., 2003). The expression of CA-Rho and DN-Rho in migrating EP cells would also be informative: by the simplest version of model that MsEphrin reverse signaling involves the local activation of Rho in filopodia that grow onto the midline cells, CA-Rho would be predicted to inhibit migration and outgrowth, while DN-Rho would be predicted to generate midline crossover phenotypes.

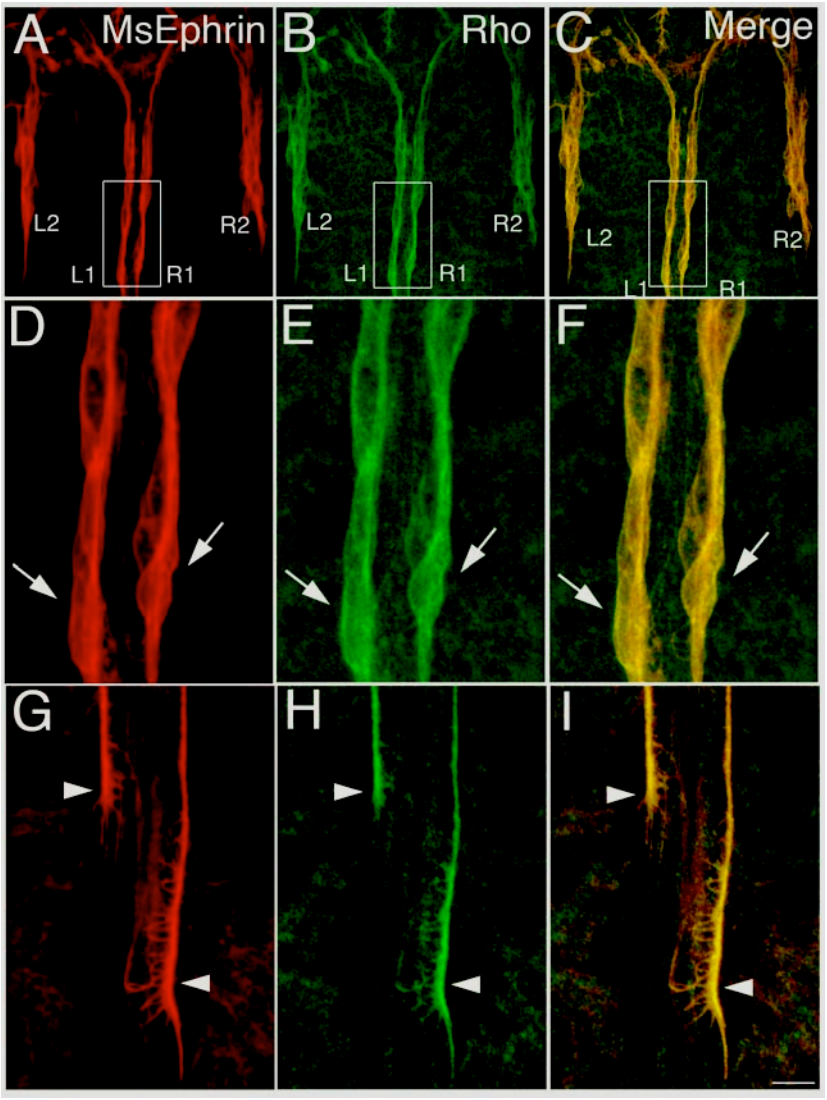

Figure 3. MsEphrin (red) colocalizes with Rho1 (green) during EP cell development. *A-I*: An embryo at 65% of development was immunostained with antibodies against MsEphrin and Rho1 (p1D9; from the Developmental Studies Hybridoma Bank; 1:100). *A-C*: A low-magnification image showing that Rho1 expression, like MsEphrin, is restricted to the EP cells. Rho1 is also expressed by the EP cells that migrate ventrally (not shown). *D-F*: A high-magnification image representing the boxed region in A-C. MsEphrin and Rho1 colocalize in the EP cells' somata (see arrows), although the pattern of Rho1 is less uniform (more punctate) than MsEphrin. *G-I*: Like Ephrin, Rho1 is distributed to the distal portions of the EP cells' leading processes (see arrowheads). Scale bar = 40 μm for A-C; 8 μm for D-I. In A-C, L = left; R = right.

An important consideration in these proposed studies is, why investigate a pathway that seems to have been exhaustively investigated in other systems? One

important reason is that most previous investigations have either been conducted in vitro or at a level of resolution that precluded an analysis in individual neurons in their normal developmental context. In addition, an investigation into the potential role of Rho in the migrating EP cells may help distinguish between our model (that predicts filopodial retraction) and published models based on Src-dependent stimulation of cell adhesion via FAK-dependent changes in integrin adhesion (described above).

Possible roles of MsEphrin that are independent of MsEph receptors.

As illustrated in chapters 2 and 3, MsEph receptor expression in the ENS is restricted to the midline of the midgut. However, I have observed MsEphrin immunoreactivity in neurons throughout the ENS that ultimately occupy positions that are spatially distant from the midline, such that it is highly unlikely that they make contact with cells that express MsEph. Some examples include the EP cells that travel along the more lateral muscle bands on the midgut (L2, L3, R2, and R3; (see chapter 2, Fig.1), as well as the EP cells that travel anteriorly from the foregut/midgut boundary along radial muscles on the foregut (see the asterisks in Fig. 2.5). MsEphrin and pSrc antibodies also co-label neurons in the frontal ganglion and hypocerebral ganglion located more rostrally on the esophagus (not shown are three foregut neurons named fn1, fn2, and fn3) (Wright et al., 1998). Fn3 is shown at the top of panels A-C in figure 4. Intriguingly, MsEphrin and pSrc are also detected within a population of bundled axons (of unknown origin) that run as a distinct fasciculated bundle within the esophageal nerve (EN; asterisks, Fig. 5.4 A-C). Branches from these axons can also be traced laterally onto the foregut where they

elaborate terminal processes (Fig. 5.4 D-F). Again, no MsEph receptor expression has been detected in the vicinity of these neurons or their terminals.

There are several possible explanations for this apparent discrepancy in MsEphrin and MsEph receptor distributions. First, it is possible that in all neurons of the ENS are simply programmed to express MsEphrin, independent of their eventual position along the gut musculature. Alternatively, MsEphrin may have roles that are independent of MsEph receptors, possibly being activated by unknown factors in *cis* or in *trans*. Surprisingly, there is precedent for Eph receptor-independent activities of Ephrins, as shown by epistasis experiments in *C. elegans* (Chin-Sang et al., 2002). *C. elegans* expresses three GPI-linked Ephrin ligands (named EFN1, 2 and 3), which function as ligands for VAB-1, an Eph receptor homolog (Chin-Sang et al., 1999). While single or double mutants of the three Ephrin genes have essentially no phenotype, efn1/2/3 triple mutants phenocopy vab-1 mutants, which have a variety of defects that lead to lethality (Wang et al., 1999). It has thus been suggested that EFN1,2, and 3 may act redundantly and are each dependent on VAB1. However, *C. elegans* encodes a fourth Ephrin homolog, EFN4, which is expressed by many cells that do not make contact with cells that express VAB-1 (Chin-Sang et al., 2002). Efn4/vab-1 double mutants have phenotypes (gastrulation defects) that are much more severe than in vab-1 single mutants, suggesting that EFN4 may have VAB-1 independent roles.

An experiment that might reveal MsEph-independent functions for MsEphrin in the developing ENS would be to test the effects of our MsEphrin-specific morpholinos on the differentiation of neurons that never contact MsEph-expressing target cells. Previously, MsEphrin morpholinos were demonstrated to knock down MsEphrin protein expression in the foregut neurons (fn1, 2, & 3) as well as in the EP cells (T.M.C., unpublished results),

but an independent marker was not available for analyzing morphological changes that may have occurred in these neurons following the loss of MsEphrin. However, as demonstrated previously by Wright et al (Wright et al., 1998) anti-cGMP antibodies strongly label these neurons following treatment with nitric oxide donors, and a new source for these antibodies has recently become available (a kind gift of D.B. Morton, Oregon Health & Sciences University). Therefore, a morpholino-based knock-down of MsEphrin expression could be coupled with an analysis of the effects of this manipulation on the foregut neurons, using cGMP immunohistochemistry to analyze their growth and differentiation. If any morphological phenotypes are observed in these neurons, this would provide compelling evidence for an MsEphrin-dependent function that is independent of MsEph receptor activity, at least in some circumstances. The results of this experiment might therefore provide the foundation for additional experiments to investigate whether MsEphrin can also activate Src independent of MsEph receptor-dependent interactions (given my observation that MsEphrin and MsSrc colocalize in these neurons; Fig. 4). It will also be interesting to investigate possible factors that may activate MsEphrin in *cis* or in *trans*.

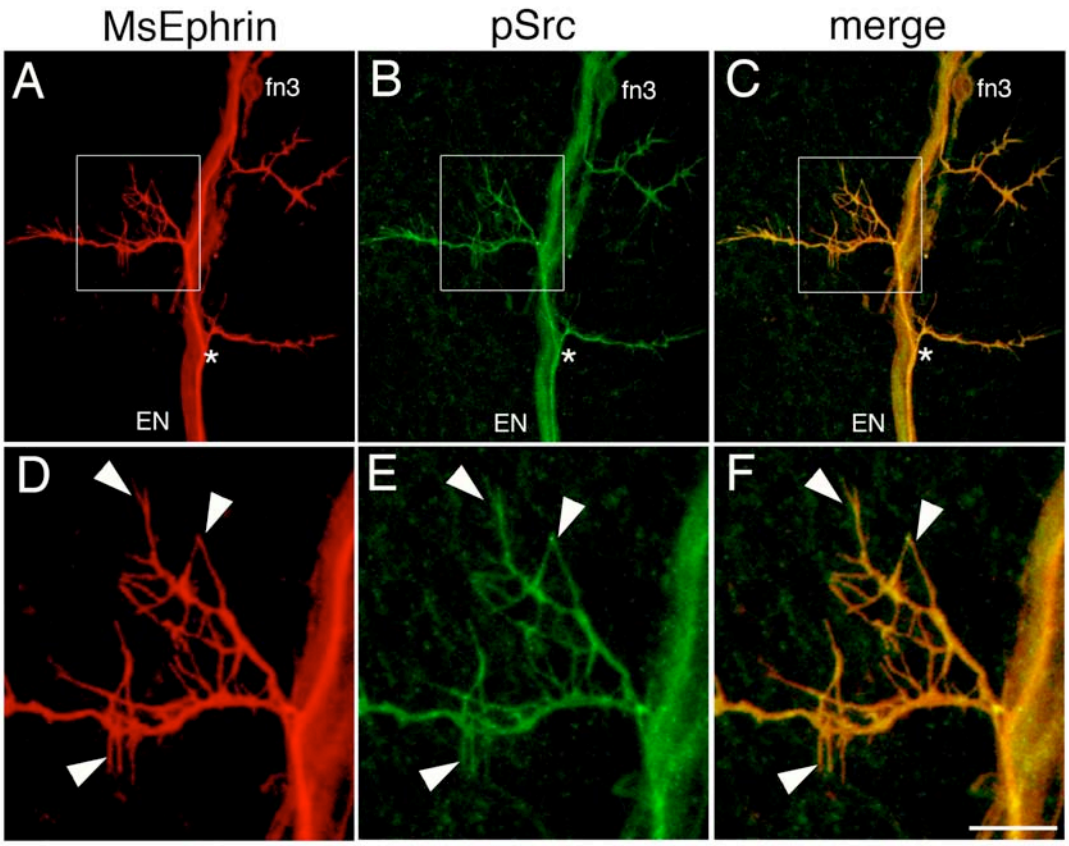

Figure 4. MsEphrin and pSrc colocalize in foregut neurons that do not apparently make contact with cells expressing MsEph receptors. An embryo at 60% of embryonic development was immunostained with antibodies against MsEphrin (red) and pSrc (green); a region of the foregut is shown where MsEph receptors are not detectably expressed. *A-C*: low magnification image showing that MsEphrin and pSrc are co-expressed at particularly high levels in a fasciculated bundle of axons within the esophageal nerve (EN), and in the fn3 (an identified foregut neuron). The strongly labeled axon fascicle in the EN may originate from neurons in the frontal ganglion (not shown). *D-F*: high magnification image from the boxed region in A-C. MsEphrin and pSrc colocalize in terminal branches projecting from these axons onto the foregut musculature (arrowheads). Scale bar in F = 40 μm for A-C; 10 μm for D-F.

The reconciliation of adhesion with filopodial repulsion: Investigations into Kuzbanian-mediated cleavage of MsEphrin.

As discussed in the introductory chapter, one paradox of Ephrin-Eph receptor interactions is that these high affinity receptor-ligand interactions often result in cell-cell repulsion. For Ephrin-As, a secretase-dependent mechanism has been proposed to regulate this process, whereby interactions between EphA receptors and Ephrin-A ligands stimulate cleavage of the Ephrin ligand in *cis* by the metalloprotease Kuzbanian (a.k.a. ADAM 10)(Hattori et al., 2000). A more recent report suggests that ligand cleavage may also occur in *trans*, under circumstances where Kuzbanian and Ephrin-As are expressed on the membranes of opposing cells (Janes et al., 2005), although this type of ADAM-dependent cleavage from one cell to another has not been documented in other contexts.

Interestingly, in preliminary studies using riboprobes against the *Manduca* ortholog of Kuzbanian (MsKuz), we used whole-mount *in situ* hybridization histochemistry to show that like MsEphrin, MsKuz is expressed by the EP cells during all phases of their development. Fig. 5 shows embryos at 65% of development that were labeled with antisense riboprobes against either MsEphrin- or Kuzbanian. Notably, Kuzbanian does not appear to be expressed by the midline cells. This pattern of co-expression suggests that MsEphrin, which is GPI-linked, may indeed be subject to Kuzbanian-mediated cleavage (in *cis*) upon interaction with midline MsEph receptors. It will be important first to conduct biochemical investigations (*in vitro*) to determine whether Kuzbanian can cleave MsEphrin: for example, by co-expressing the two proteins in tissue culture cells and monitoring for the presence of cleaved MsEphrin fragments after treatment with MsEph-Fc. Alternatively, MsEph could be expressed in a

second population of culture cells, allowing the effects of MsEph-MsEphrin interaction *in trans* to be tested by this assay. Conversely, if Kuzbanian normally cleaves MsEphrin after contact with MsEph receptors, inhibiting this process would be predicted to cause the EP cells to aberrantly adhere to the midline, possibly stalling the neurons and their processes or even permitting ectopic crossover events. Kuzbanian-specific morpholinos or pharmacological inhibitors targeting ADAM secretases could be applied in embryo culture, followed by an analysis of the extent and directionality of EP cell migration, outgrowth, and synapse formation (chapters 3 and 4; Wright et al., 1998). One caveat to these approaches is that Notch-mediated functions would also potentially be disrupted in these experiments (Kuzbanian also cleaves Notch); thus, it would be important to control for any side effects related to Notch inhibition.

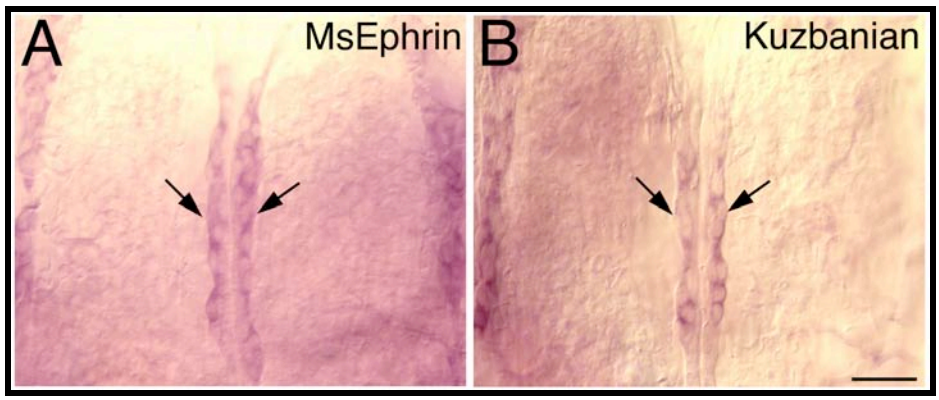

Figure 5. Kuzbanian mRNA is expressed by the EP cells during migration and outgrowth. A: an embryo at 65% of development was fixed and probed with an MsEphrin-specific antisense riboprobe (1:100; see chapter 2, Fig. 2), which labels the EP cells (see arrows). B: an identically-staged embryo was probed with a Kuzbanian riboprobe (1:50; probe A10g) and shows a similar pattern of staining as the MsEphrin--specific probe. Scale bar = 40 μm.

The balance of adhesion and repulsion: Src as a possible integrator.

In a migratory neuron, how are multiple guidance cues integrated such that their net influence is directed motility along an appropriate pathway? For example, as the EP cells migrate posteriorly along the muscle bands, they must balance MsFas II-dependent adhesion (with MsFas II on the bands) with MsEphrin-dependent repulsion (mediated by MsEph receptors on the adjacent midline muscles). Preliminary biochemical data suggests MsEphrin- and MsFas II-mediated signals may converge on the regulation of Src. Single gut explants were probed with either 25 μg/ml Fc or MsEph-Fc for 45 min, and phosphorylated Src levels were then measured in these samples on a Western blot (following the methods outlined in chapter 4; Fig. 6). In some samples, MsEph-Fc was also added in combination with either low or high concentrations of a second fusion protein consisting of the extracellular domain of MsFas II and alkaline phosphatase (MsFas II-AP), which should target endogenous MsFas II on the EP cells via homophilic binding (c.f. Goridis and Brunet, 1992; Rao et al., 1992). As described in chapter 4, MsEph-Fc alone led to a dramatic increase in Src phosphorylation in single gut explants, compared to no detectable stimulation by Fc alone (Fig. 6, lanes 1-2). However, when MsFas II-AP was added together with MsEph-Fc, I observed substantially less Src phosphorylation, compared with the samples treated with MsEph-Fc alone (Fig 6, lane 3). Indeed, at the highest concentration of MsFas II-AP, the stimulation of Src phosphorylation by MsEph-Fc was almost completely abolished.

There are a number of potential caveats to this experiment that need to be addressed in future studies. For one, it is possible that MsFas II-AP interacts with

MsEph-Fc when applied simultaneously, thereby simply inhibiting the ability of MsEph-Fc proteins to bind to MsEphrin on the EP cells. Applying the two proteins sequentially rather than simultaneously would allow us to control this type of *in vitro* interaction; pull-down assays (with anti-Fc or anti-AP antibodies) could also be used to monitor for interactions between MsEph-Fc and MsFas II-AP in solution. Mixtures of MsFas II and MsEphrin with Fc fragments and other fusion proteins would also provide additional controls for the specificity of their combined effects. Given the possibility that both MsEphrin and MsFas II might both be cleaved by endogenous secretases (like MsKuz), this type of interaction might even reflect a physiological interaction between the two proteins in the context of the developing ENS, although such a mechanism has not previously been described for Ephrins. An alternative, and perhaps more appealing interpretation is that activating endogenous MsFas II on the EP cells (via homophilic interactions with MsFas II on the muscle bands) normally down-regulates MsSrc activity, providing a local mechanism to promote the adhesion and outgrowth of filopodia that have extended along the correct pathways. By this scenario, treating the migrating EP cells with MsFas II-AP fusion proteins would be expected to decrease Src phosphorylation (as observed in lanes 3 and 4), in contrast to the effects of MsEphrin reverse signaling. In this manner, local differences in the level of MsSrc activity would provide a mechanism for integrating input from guidance cues that induce opposing effects on EP cell guidance. In the future, it will be necessary to determine if MsFas II-AP alone can reduce endogenous levels of pSrc, and whether exposing the migratory EP cells in cultured embryos with a combination of MsEphrin and MsFas II constructs provides a means of inducing modulated changes in MsSrc activity. If MsFas II and

MsEphrin can be shown to have opposing effects on Src phosphorylation within the ENS, this system will provided a powerful set of assays for investigating the molecular mechanisms by which migrating neurons integrate different cues during directed migration within the context of a developing embryo.

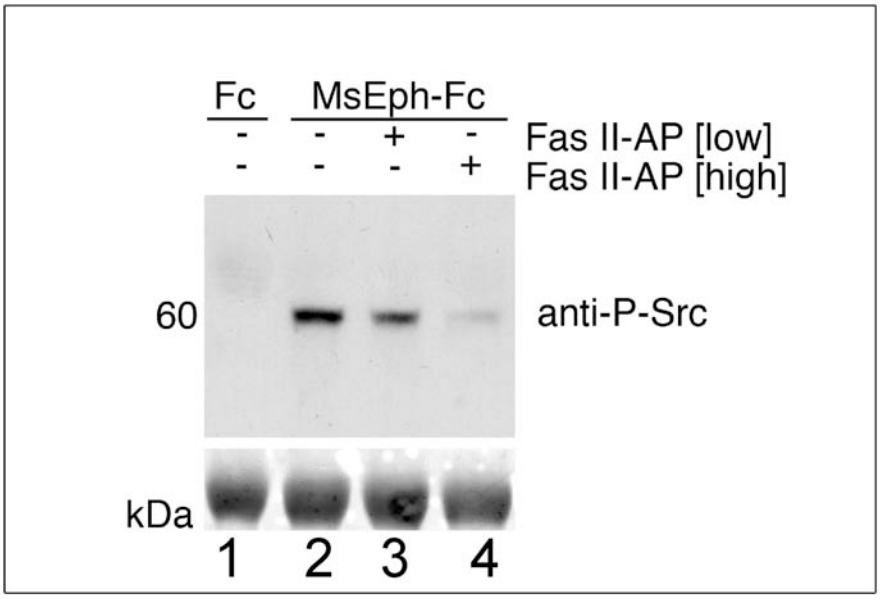

Figure 6. The extracellular domain of MsFas II inhibits Src phosphorylation. ENS explants were probed with different combinations of Fc fusion proteins and the extracellular domain of MsFas II (MsFas II-AP). (Lane 1) An individual gut was treated with Fc alone and showed no increase in pSrc. (Lane 2) MsEph-Fc alone leads to a characteristic increase in Src phosphorylation. (Lanes 3 and 4) The addition of MsEph-Fc to MsFas II-AP in low and high concentrations shows a dose-dependent decrease in Src phosphorylation. The lower panel represents Ponceau S staining of an unknown band on the Western blot as a loading control.

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