Development/Plasticity/Repair

Reverse Signaling by Glycosylphosphatidylinositol-Linked *Manduca* Ephrin Requires a Src Family Kinase to Restrict Neuronal Migration *In Vivo*

Thomas M. Coate, Tracy L. Swanson, and Philip F. Copenhaver
Department of Cell and Developmental Biology, Oregon Health & Science University, Portland, Oregon 97239

Reverse signaling via glycosylphosphatidylinositol (GPI)-linked Ephrins may help control cell proliferation and outgrowth within the nervous system, but the mechanisms underlying this process remain poorly understood. In the embryonic enteric nervous system (ENS) of the moth *Manduca sexta*, migratory neurons forming the enteric plexus (EP cells) express a single Ephrin ligand (GPI-linked MsEphrin), whereas adjacent midline cells that are inhibitory to migration express the cognate receptor (MsEph). Knocking down MsEph receptor expression in cultured embryos with antisense morpholino oligonucleotides allowed the EP cells to cross the midline inappropriately, consistent with the model that reverse signaling via MsEphrin mediates a repulsive response in the ENS. Src family kinases have been implicated in reverse signaling by type-A Ephrins in other contexts, and MsEphrin colocalizes with activated forms of endogenous Src in the leading processes of the EP cells. Pharmacological inhibition of Src within the developing ENS induced aberrant midline crossovers, similar to the effect of blocking MsEphrin reverse signaling. Hyperstimulating MsEphrin reverse signaling with MsEph-Fc fusion proteins induced the rapid activation of endogenous Src specifically within the EP cells, as assayed by Western blots of single embryonic gut explants and by whole-mount immunostaining of cultured embryos. In longer cultures, treatment with MsEph-Fc caused a global inhibition of EP cell migration and outgrowth, an effect that was prevented by inhibiting Src activation. These results support the model that MsEphrin reverse signaling induces the Src-dependent retraction of EP cell processes away from the enteric midline, thereby helping to confine the neurons to their appropriate pathways.

Introduction

During the formation of the nervous system, Ephrin–Eph receptor interactions regulate multiple aspects of cell growth, migration, and differentiation (Wilkinson, 2001; Kullander and Klein, 2002; Pasquale, 2005). In vertebrates, Eph receptors constitute the largest family of receptor tyrosine kinases and are classified into two groups: EphA receptors preferentially bind glycosylphosphatidylinositol (GPI)-anchored type-A Ephrins, whereas EphB receptors typically bind transmembrane type-B Ephrins (Pasquale, 2004, 2005). However, determining the function of specific Ephrins and Ephs *in vivo* has been complicated by the discovery that multiple ligand–receptor combinations are often expressed by neurons and can interact promiscuously (Himanen et al., 2004). In addition, whereas Ephrin ligands stimulate conventional "forward" signaling by activating Eph receptors, Eph receptors can also induce "reverse" signaling via their cognate Ephrin ligands, although the relative importance of forward versus reverse signaling may vary substantially in different contexts (Kullander and Klein, 2002; Egea et al., 2005). Reverse signaling via Ephrin-B ligands contributes to a variety of morphogenetic events, including segmentation, axonal guidance, and synaptogenesis (Wilkinson, 2001; Davy and Soriano, 2005). In many instances, Ephrin-B reverse signaling leads to the activation of Src family kinases (SFKs), although a number of other signaling cascades have also been associated with this process (Kullander and Klein, 2002). Several examples of reverse signaling via Ephrin-A ligands have also now been documented (Davy and Robbins, 2000; Huai and Drescher, 2001; Holmberg et al., 2005), including *in vitro* studies linking this process to the local recruitment of SFKs (Davy et al., 1999; Huai and Drescher, 2001; Lim et al., 2008). However, the signaling mechanisms that mediate Ephrin-A reverse signaling *in vivo* remain poorly understood.

Recently, we identified a novel role for reverse signaling via a GPI-linked Ephrin in the enteric nervous system (ENS) of the tobacco hornworm, *Manduca sexta*. As in other insects, *Manduca* expresses only one Ephrin (GPI-linked MsEphrin) and one Eph receptor (MsEph), which has facilitated investigations into their normal functions (Kaneko and Nighorn, 2003; Coate et al., 2007). Using an embryonic culture assay that provides access to the ENS, we recently showed that endogenous MsEphrin–MsEph receptor interactions prevent migratory enteric neurons (EP cells) from growing aberrantly across the enteric midline (Coate

Received Nov. 12, 2008; revised Jan. 20, 2009; accepted Jan. 26, 2009.

We thank Drs. Doris Kretzschmar, David Morton, and Larry Sherman for critical input on this manuscript. We also thank Drs. Zak Wills and Michael Greenberg (Children's Hospital, Harvard University, Boston, MA) for generously providing their EphB2 antibodies and Drs. Kara Johnson and Brian J. Druker (Oregon Health & Science University) for antibodies against pSrc and Src used in our pilot investigations. The monoclonal antibody E7, developed against β-tubulin by Dr. Michael Klymkowsky, was obtained from the Developmental Studies Hybridoma Bank (maintained by the Department of Biological Sciences, University of Iowa, Iowa City, IA), under the auspices of the National Institute of Child Health and Human Development.

Correspondence should be addressed to Dr. Philip F. Copenhaver, Department of Cell and Developmental Biology L-215, Oregon Health & Science University, 3181 Southwest Sam Jackson Park Road, Portland, OR 97239. E-mail: copenhav@ohsu.edu.

DOI:10.1523/JNEUROSCI.5464-08.2009

 0270-6474/09/293404-15$15.00/0

et al., 2008). Notably, we found that this response is mediated by reverse signaling through MsEphrin on the EP cells, whereas forward signaling by MsEph receptors plays no discernable role in ENS development. However, the signaling mechanisms by which MsEphrin restricts the behavior of these migratory neurons remained undefined.

We have now investigated the role of SFKs in mediating MsEphrin reverse signaling within the developing ENS. We show that the induction of MsEphrin reverse signaling stimulates endogenous Src in the EP cells, which is required for the neurons to respond to MsEph receptors at the enteric midline. Conversely, inhibiting Src mimics the effects of blocking MsEphrin reverse signaling, allowing the neurons to cross the midline. Last, our results demonstrate an atypical role for SFKs in the control of neuronal motility, whereby MsEphrin-dependent activation of Src leads to the local inhibition of neuronal outgrowth.

Materials and Methods

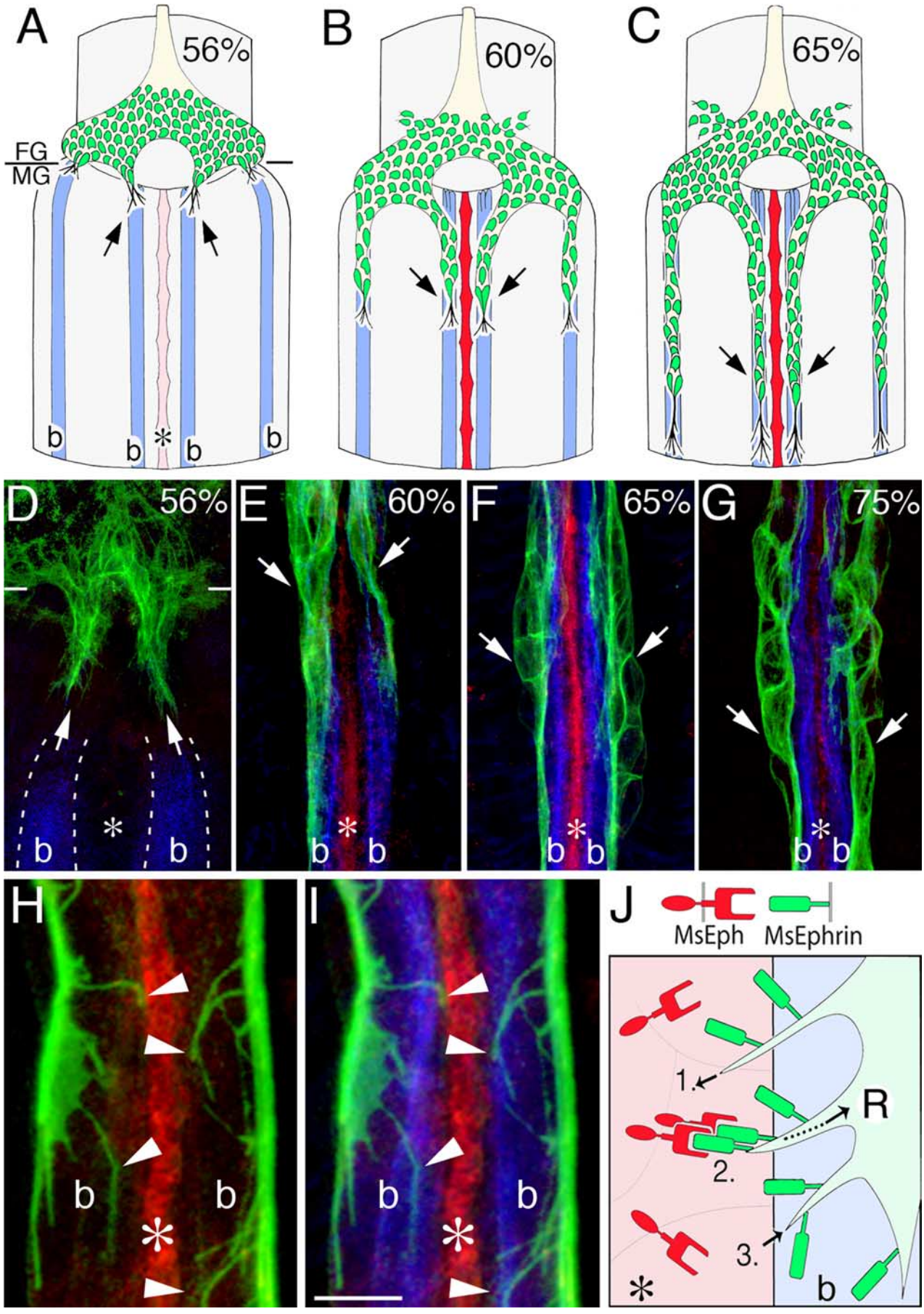
Embryo preparation and immunostaining. *Manduca sexta* embryos were collected from an in-house breeding colony and staged according to standard internal and external markers (1 h at 25°C = 1% of development) (Copenhaver and Taghert, 1989b). All reagents were from Sigma-Aldrich, unless noted otherwise. Embryos were dissected in defined saline (in mM: 140 NaCl, 5 KCl, 28 glucose, 40 $CaCl_2$, and 5 HEPES, pH 7.4) (Horgan and Copenhaver, 1998; Coate et al., 2008) and fixed in 4% paraformaldehyde, pH 7.4 (Electron Microscopy Sciences) for 1 h at room temperature (RT). Embryos were then rinsed and permeabilized with PBS plus 0.6% Triton X-100 (PBST) and incubated for at least 1 h in blocking solution (PBST plus 10% normal serum and 0.1% sodium azide). For preparations immunostained with antibodies against phosphorylated Src [to label tyrosine kinase orthologous to the Rous sarcoma virus oncogene protein pp60 (v-Src); see below], a 1% solution of bovine serum albumin (BSA; immunoanalytical grade; Promega) was used instead of normal serum as a blocking agent, to avoid exposure to serum-derived phosphatases (Garcia et al., 2004). Primary antibodies were then diluted in blocking solution and applied to the embryos for 2 h at RT or overnight at 4°C at the following concentrations: mouse anti-*Manduca* fasciclin II, 1:20,000 (anti-MsFas II, monoclonal C3) (Wright et al., 1999); mouse anti-Neuroglian, 1:500 (3B11) (Chen et al., 1997); mouse anti-Src, 1:1000 (7G9; Cell Signaling Technology); and mouse anti-phosphorylated Src, 1:100–1:500 (pSrc, anti-pY416; Cell Signaling Technology). To detect MsEph, we used an affinity-purified antibody (at 1:200) that recognizes an evolutionarily conserved epitope shared by vertebrate EphB2 and MsEph, as described previously (Coate et al., 2008), which was generously provided by Dr. M. E. Greenberg and colleagues (Children's Hospital, Boston, MA) (Dalva et al., 2000). To detect MsEphrin, unfixed embryos were incubated for 90 min at RT with anti-MsEphrin (1:100), postfixed with 4% paraformaldehyde (for 1 h at RT), and labeled with fluorochrome-conjugated secondary antibodies (Coate et al., 2007, 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) were used at 1:400–1:800. Preparations were imaged on a Bio-Rad 1024 ES laser-scanning confocal microscope in the Live Cell Imaging Facility in the Center for Research on Occupational and Environmental Toxicology (Oregon Health & Science University), with the assistance of Dr. S. Kaech-Petrie. *Z*-stack images were then flattened, pseudo-colored, and uniformly adjusted for brightness and contrast, as needed, in Photoshop (Adobe Systems).

Immunoblots of embryonic tissues. Tissue samples from staged embryos were dissected, frozen on dry ice, and homogenized in Laemmli buffer (Laemmli, 1970) following our published methods (Swanson et al., 2005). Protein extracts (25 μg per lane) were separated by electrophoresis in 10% polyacrylamide gels under reducing conditions and transferred to nitrocellulose. Immunoblots were preblocked for 1 h using Blotto B solution (Rockland Immunochemicals), incubated with anti-Src (1:2000) or anti-pSrc (1:1000) antibodies overnight at 4°C, rinsed, and incubated with secondary antibodies conjugated to horseradish peroxidase (HRP; Jackson ImmunoResearch). Bound antibody was visualized using West-Pico chemiluminescence substrates (Pierce).

In vivo *manipulations of cultured embryos.* For overnight cultures, embryos were isolated at 52–53% of development (shortly before the onset of EP cell migration at 55%) in sterile embryonic culture medium (Horgan et al., 1995). Embryos were gently restrained in Sylgard-coated chambers, and the developing ENS was exposed by a small incision in the dorsal body wall. The EP cells were then treated with culture medium containing the Src inhibitor 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine (PP2; Calbiochem), or its inactive analog 4-amino-7-phenylpyrazolo[3,4-d]pyrimidine (PP3), or with medium containing matched concentrations of DMSO (up to 0.1% v/v as a vehicle control). For some experiments, an additional group of embryos was treated with the Src inhibitor SU6656 (10 μM; Calbiochem). The cultures were allowed to develop for another 24 h at 28°C, redissected to expose the ENS, fixed, and immunostained with anti-MsFas II antibodies to reveal the complete distributions of the neurons and their processes on the foregut and midgut. For these studies, the ABC–HRP method (Vector Laboratories) was used to detect MsFas II immunoreactivity, using diaminobenzidine with H_2O_2 as a substrate. The extent of EP cell migration, axon outgrowth, and midline crossing events were quantified using camera lucida and photomicrographic techniques, as described previously (Wright et al., 1999; Coate et al., 2008).

To stimulate reverse signaling via endogenous MsEphrin in the EP cells, dimeric MsEph-Fc fusion proteins were generated by our published methods (Coate et al., 2007, 2008) and applied to the developing ENS in the presence of 0.1% DMSO (to match other experimental conditions). Control Fc (human; Jackson ImmunoResearch) or monomeric MsEph-6His fusion proteins (which bind MsEphrin ligands but do not activate reverse signaling) were used as controls, as described previously (Coate et al., 2008). For assays of filopodial orientation, the developing ENS was exposed in embryos at 60% of development (a period of active EP cell migration and outgrowth) and treated with 10 μM PP2, 10 μM SU6656, or vehicle control for 3 h at 28°C. The preparations were then immunostained with anti-MsFas II antibodies plus Alexa-fluor 488-conjugated secondary antibodies and visualized by confocal microscopy; 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 enteric midline regions. Morphometric measurements of the leading processes were performed as described previously (Coate et al., 2008). Some of the preparations in these experiments were also exposed to DMSO or PP2 for 1 h at 28°C and immunostained with anti-pSrc antibodies (detected with anti-rabbit 488-conjugated secondary antibodies) to determine the extent to which PP2 inhibited Src phosphorylation *in vivo*. Confocal *Z*-stack images were then acquired in the region of the EP cell somata (or equivalent control regions) under linear parameters; identical laser and acquisition settings were used to image each preparation. Levels of fluorescent intensity in the EP cells versus adjacent control regions were quantified using ImageJ software.

MsEph morpholino experiments. Morpholino antisense oligonucleotides (morpholinos; Gene Tools) were designed against the 5′ untranslated region (UTR) and coding domain of the MsEph receptor. A morpholino directed against the 5′ UTR sequence flanking the initiation codon of the MsEph gene (5′-GTTTTTGGTGCGTGTCGTGTGTGAT-3′) proved optimally effective at knocking down MsEph receptor expression without affecting other proteins (described below). We also routinely treated matched sets of preparations with the standard control morpholino from Gene Tools (5′-CCTCTTACCTCAGTTACAATTTATA-3′), which does not correspond to any insect gene sequence in the NCBI database and has no detectable biological activity in *Manduca* embryos (Coate et al., 2008; and our unpublished observations). In some experiments, we also used a second morpholino targeting an upstream (5′) region of the MsEph sequence (5′-CGATCACCGCTAACCACATCATCGC-3′) that was ineffective at knocking down MsEph receptor expression and was used as a control for nonspecific and off-target effects. Additional controls included a morpholino specific for *Manduca* APPL (amyloid precursor protein-like protein) (5′-CGTTGCTTCCCCGACT-


A
56%
FG
MG
b
b
*
b
b
B
60%
C
65%
D
56%
E
60%
F
65%
G
75%
H
I
J
MsEph
MsEphrin
1.
2.
3.
R

CTGGTAGGA-3′) and a missense control morpholino (5′CCGCGTTGCTTCCCACCAGCCCGG-3′), neither of which affected MsEph receptor expression (data not shown).

Initial tests of the effectiveness and specificity of each morpholino were performed using *Manduca* GV1 cells (Lan et al., 1999; Hiruma and Riddiford, 2004), an ectodermal cell line that endogenously expresses MsEph. GV1 cells were grown in Grace's complete medium plus 10% fetal bovine serum, and morpholinos were delivered into replicate GV1 cell cultures in 24-well plates in the presence of serum using 0.6% Endo-Porter, as per the manufacturer's guidelines (Summerton, 2005). After 48 h, lysates from morpholino-treated cell cultures were analyzed for MsEph expression levels in Western blots (using anti-EphB2 antibodies at 1:500). For loading controls, an anti-β-tubulin monoclonal antibody (E7 supernatant; Developmental Studies Hybridoma Bank) was used at 1:50. Relative levels of MsEph expression were then visualized and quantified in triplicate, using the Odyssey infrared imaging system (LI-COR Biosciences).

To knock down MsEph receptor expression within the developing ENS, MsEph morpholinos or a variety of control morpholinos (1–50 μM) were diluted in defined saline (supplemented with 0.5% penicillin/streptomycin, 0.2% ecdysone, 0.1% insulin, and 0.2 M L-glutamine) and delivered into the EP cells of cultured embryos with 0.6% Endo-Porter (Coate et al., 2008). We routinely included 10% heat-inactivated normal horse serum in all cultures treated with either control or experimental morpholinos, which is compatible with the Endo-Porter delivery system (Summerton, 2005) and enhanced the viability of embryos grown in culture (our unpublished observations). To optimize the effectiveness of this protocol, embryos were treated with morpholinos starting at 48% of development (±1%; ~7 h before the onset of EP cell migration) and then allowed to develop for another 48 h at 28°C (through the completion of EP cell migration and outgrowth). At the end of each experiment, the preparations were immunostained with anti-MsFas II, and midline crossing events were quantified as described previously (Coate et al., 2008).

Single gut explant assays. Staged embryos at 60–62% of development were dissected in defined saline (supplemented with 4 mM $CaCl_2$ and ~0.5% BSA). The intact midgut of each animal was then explanted by incising the surrounding trachea and adjacent regions of the foregut and hindgut. Single embryonic gut segments (~400 μm long by 100 μm diameter) were transferred by micropipette into individual Eppendorf tubes containing 10 μl of defined saline plus 25 μg/ml dimeric MsEph-Fc, monomeric MsEph-6His, or human Fc as a control. This concentration was based on our previous demonstration that MsEph-Fc but not MsEph-6His can stimulate MsEphrin-dependent reverse signaling in cultured embryos (Coate et al., 2008). In some experiments, isolated gut explants were pretreated with 10 μM PP2 or PP3 for 20 min, before the addition of MsEph fusion proteins. After a 1–15 min incubation at RT, the samples were transferred onto dry ice to halt additional signaling activity. Individual gut explants were then homogenized in 10 μl chilled 2× lysis buffer, and the lysate from each midgut was electrophoresed in separate lanes on 10% polyacrylamide gels. Protein samples were transferred to nitrocellulose membranes, blocked using Blotto B solution, and probed with anti-pSrc antibodies (1:1000) overnight at 4°C, followed by visualization with anti-rabbit secondary antibodies, as described above. The blots were then stripped and reprobed with anti-Src antibodies to control for variability between sample groups.

In vivo Src activation, imaging, and quantification. Embryos at 60–62% of development were dissected in defined saline 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) for 25 min at RT. In some experiments, the preparations were pretreated with PP2 or PP3 for 20 min, before the addition of the fusion proteins. At the end of the incubation period, the embryos were fully dissected, fixed in chilled 4% paraformaldehyde for 1 h, and rinsed extensively in PBST. After preblocking for 2 h in PBST plus 1% BSA, they were immunostained with anti-MsFas II and anti-pSrc antibodies at a reduced concentration (1:500) to enhance the sensitivity of this assay, followed by incubation with fluorochrome-conjugated secondary antibodies for exactly 1 h. Confocal *Z*-stack images of the axons and leading processes of the EP cells were acquired under linear parameters; identical laser and acquisition settings were used for all experiments.

For quantifying fluorescence intensities associated with EP cell growth cones, two of the eight *Z*-stack sections (encompassing the majority of their leading filopodia on two dorsal band pathways) were flattened using MetaMorph Imaging software (Molecular Devices), 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 growth cone. For each condition, ≥10 growth cones and >100 filopodia were analyzed. Fluorescent intensity values were acquired using ImageJ software (http://rsb.info.nih.gov/ij/). Statistical analyses were performed using Student's two-tailed *t* test to compare means; sample sizes were based on the number of growth cones analyzed per condition.

←

Figure 1. MsEphrin ligands and MsEph receptors are expressed in a cell-type-specific manner within the developing ENS of *Manduca*. ***A–C***, Summary of EP cell migration during the formation of the enteric plexus [spanning the foregut (FG) and midgut (MG) boundary]. ***A***, At ~56% of development, subsets of the EP cells on the foregut (which express MsEphrin; green) align with one of eight muscle band pathways (b; dark blue) that have formed on the adjacent midgut surface; only the four dorsal muscle bands are shown. ***B***, By ~60% of development, groups of EP cells have begun to migrate posteriorly along each band, extending exploratory filopodia onto the bands and adjacent interband muscles of the midgut; smaller subsets migrate onto radial muscles on the foregut (radial muscles not shown). Concurrently, midline muscles on the dorsal and ventral surface of the midgut begin to express the MsEph receptor (red; only the dorsal midline is shown). ***C***, By ~65% of development, the EP cells have completed their migration but continue to extend axons along the band pathways while avoiding the midline. ***D–G***, Whole-mount immunostained embryos illustrate the developmental regulation of MsEph receptor expression in the ENS. ***D***, At 56% of development, when the EP cells (expressing MsEphrin; green) begin migrating onto the muscle band pathways (expressing Neuroglian; blue), MsEph receptors are not yet detectable in the adjacent midline cells (asterisk). White hatchmarks show the foregut–midgut boundary. ***E***, By 60% of development, the EP cells are migrating rapidly onto the bands, and MsEph receptor expression is now clearly evident in the midline cells (red). ***F***, By 65% of development, the EP cells have progressed from migration to axon outgrowth along the bands, whereas MsEph levels in the midline cells remain high. ***G***, By 75% of development, MsEph levels have substantially diminished, although the EP cells (and their processes) continue to express MsEphrin throughout embryogenesis. ***H***, ***I***, Magnified view of EP cell growth cones on the paired dorsal muscle bands at 65% of development (posterior to the region shown in ***F***). Although the leading processes remain primarily confined to the muscle band pathways (labeled with anti-Neuroglian in ***I***; blue), MsEphrin-positive filopodia (green) grow up to the midline cells expressing MsEph receptors (red) but then appear to stall and retract (arrowheads). ***J***, Model for the control of EP cell guidance at the enteric midline: MsEphrin-positive filopodia extended by the EP cells onto adjacent midline muscles (step 1) encounter MsEph receptors (step 2), which stimulate a local reverse signaling response (R) that causes the filopodia to retract (step 3). Consequently, the EP cells normally never cross the midline (asterisk), remaining confined to their normal band pathways (b). Asterisks indicate the dorsal midline cells of the midgut. Scale bar: ***D–G***, 20 μm; ***H–J***, 5 μm.

Results

Developmental regulation of MsEphrin and MsEph in the ENS coincides with EP cell migration

The formation of the ENS in *Manduca* requires a stereotyped sequence of neuronal migration, during which a population of ~300 undifferentiated neurons (EP cells) travel along preformed muscle band pathways on the midgut to form the enteric plexus (Fig. 1*A–C*). During this process, the EP cells express MsEphrin (Fig. 1, green) and migrate selectively on eight longitudinal muscle bands on the midgut surface (Fig. 1, blue; only the four dorsal muscle bands are shown). In contrast, MsEph receptors are only expressed by discrete sets of muscle cells at the dorsal and ventral midline of the midgut during this developmental period (Fig. 1, red); these regions are normally avoided by the EP cells and their processes (Coate et al., 2007, 2008). By 56% of development (Fig. 1*A*), the EP cells have delaminated *en masse* from a neurogenic placode in the foregut epithelium (Copenhaver and Taghert,

1990) and spread bilaterally around the foregut–midgut boundary, whereupon they align with one of the muscle bands. By 60% of development (Fig. 1*B*), subsets of EP cells have begun to migrate rapidly down each band pathway. As they migrate, each neuron also extends exploratory filopodia onto the adjacent midline muscles and more lateral interband regions of the midgut but then subsequently avoids these nonpathway domains (Copenhaver and Taghert, 1989b; Copenhaver, 2007). By 65% of development (Fig. 1*C*), the neurons have completed their migration, but they continue to extend axons along the muscle bands for another 15% of development before branching laterally to innervate the midgut musculature (Copenhaver and Taghert, 1989a,b).

Double immunostaining the developing ENS with antibodies against MsEphrin and its receptor revealed that their expression is surprisingly cell type specific, in contrast to the overlapping patterns of multiple ephrins and Eph receptors in other systems (Wang et al., 1999; Brownlee et al., 2000; Mendes et al., 2006; Egea and Klein, 2007). As noted previously, the EP cells continue to express MsEphrin throughout embryogenesis (Coate et al., 2007), whereas the MsEph receptor is only transiently expressed by the midline cells, coincident with the most active period of EP cell migration and outgrowth. As shown in Figure 1, *D–G*, the EP cells already express MsEphrin (green) when they first extend processes onto the midgut (at 56% of development) and maintain robust levels of expression throughout their subsequent phases of migration and outgrowth along the band pathways (immunostained for Neuroglian; blue). In contrast, MsEph receptor expression is undetectable in the midline cells before the onset of EP cell migration, aside from weak immunoreactivity in more posterior regions of the midline (data not shown). Subsequently, MsEph receptor expression becomes apparent specifically in the midline cells but not in the adjacent band pathways or the neurons themselves (Fig. 1*E*, 60% of development). Robust levels of expression persist in the midline cells throughout the period of EP cell migration and axonal outgrowth (Fig. 1*F*, 65% of development), during which the EP cells extend MsEphrin-positive filopodia up to but not across the MsEph-expressing midline cells (Fig. 1*H*,*I*). However, by 75% of development, MsEph receptor levels have declined to barely detectable levels (Fig. 1*G*), coinciding with the stage when the EP cells transition from axonal outgrowth along the bands to lateral branching and innervation of the adjacent gut musculature (Copenhaver and Taghert, 1989a,b). Thus, within the developing ENS, the expression of MsEphrin and its sole receptor (MsEph) is both cell type and stage specific.

Inhibiting MsEph expression in the ENS causes midline crossover phenotypes

The appearance of MsEph receptors on the midline cells precisely during the period of EP cell migration and outgrowth suggested that reverse signaling via MsEphrin might restrict the neurons from growing across these inappropriate regions (Fig. 1*J*). In support of this model, we previously showed that blocking endogenous MsEphrin–MsEph receptor interactions with monomeric fusion proteins (MsEph-6His or MsEphrin-6His) led to aberrant midline crossing by the neurons and their growing processes, whereas hyperstimulating the reverse signaling response (with dimeric MsEph-Fc) caused a global inhibition of migration and outgrowth, akin to a collapse response *in vitro* (Coate et al., 2008). Unexpectedly, these studies also indicated that forward signaling via MsEph receptors on the midline cells plays no apparent role in controlling EP cell behavior: applying MsEphrin-Fc (which stimulates MsEph receptor forward signaling in cell culture) simply caused the same midline crossover phenotypes as the monomeric fusion proteins, resulting from their perturbation of endogenous MsEphrin–MsEph receptor interactions. Based on these results, we predicted that inhibiting MsEph receptor expression by the midline muscle cells should also generate midline crossover phenotypes without affecting the overall extent of migration and outgrowth by the EP cells along their band pathways.

To test this hypothesis, we designed a panel of MsEph-specific morpholino antisense oligonucleotides and tested their ability to knock down the endogenous expression of MsEph receptors in *Manduca* GV1 cells (an ectodermal cell line that expresses many neuronal proteins, including MsEph). Treatment with one of the MsEph morpholinos (at 20 μM) almost completely eliminated MsEph protein expression in GV1 cultures after 2 d (Fig. 2*A*, lanes 3–4), in contrast to untreated control cultures (data not shown) or cultures treated with control morpholinos (Fig. 2*A*, lanes 1–2). To determine whether the MsEph-specific morpholino would also knock down MsEph receptor expression in the developing ENS, matched sets of embryos were opened in culture just before the onset of MsEph receptor expression in the ENS (~50% of development) and treated with either control or MsEph-specific morpholinos. After 24 h subsequent development, individual midguts from each set of embryos were isolated, lysed, and probed sequentially in Western blots with anti-Eph and anti-tubulin antibodies. Treatment with MsEph-specific morpholinos resulted in a significant reduction in the relative levels of MsEph receptor expression in the embryonic midguts compared with matched controls (quantified using the LI-COR Odyssey system). On average, samples treated with MsEph-specific morpholinos retained between 26 and 57% of the MsEph levels detected in control samples (average reduction of 43% $\pm$ 16% SEM; <0.02). Residual MsEph levels in these samples may have been attributable to persistent MsEph expression in the mid-ventral cells of the midgut, which are less accessible to morpholino treatments than the mid-dorsal cells (the focus of this investigation).

We also fixed a subset of embryos that had been treated with either control or MsEph-specific morpholinos for 24 h and double immunostained them for MsFas II and MsEph. Because endogenous levels of MsEph expression had begun to diminish by this stage of development (Fig. 1), residual MsEph immunoreactivity in the control preparations was variable and could only be detected with enhanced illumination settings (resulting in elevated background levels). Nevertheless, we could readily detect MsEph receptor-specific immunoreactivity on the midline cells of preparations treated with control morpholinos (supplemental Fig. 1*A*, available at www.jneurosci.org as supplemental material), whereas little or no expression was seen in preparations treated with morpholinos targeting MsEph receptor (supplemental Fig. 1*B*, available at www.jneurosci.org as supplemental material). These results indicated that this morpholino protocol could be used to knock down MsEph receptor expression at the enteric midline during the formation of the enteric plexus.

We therefore treated the ENS of cultured embryos with either control or MsEph-specific morpholinos for 48 h (starting at 50% of development) (Fig. 1*A*), to permit the neurons to complete their migration and outgrowth. The preparations were then fixed and immunostained with anti-MsFas II antibodies to reveal the final distributions of the neurons and their processes. In preparations treated with control morpholinos, the EP cells migrated and extended axons normally along the midgut muscle bands without deviating from these pathways (Fig. 2*B*). In contrast,

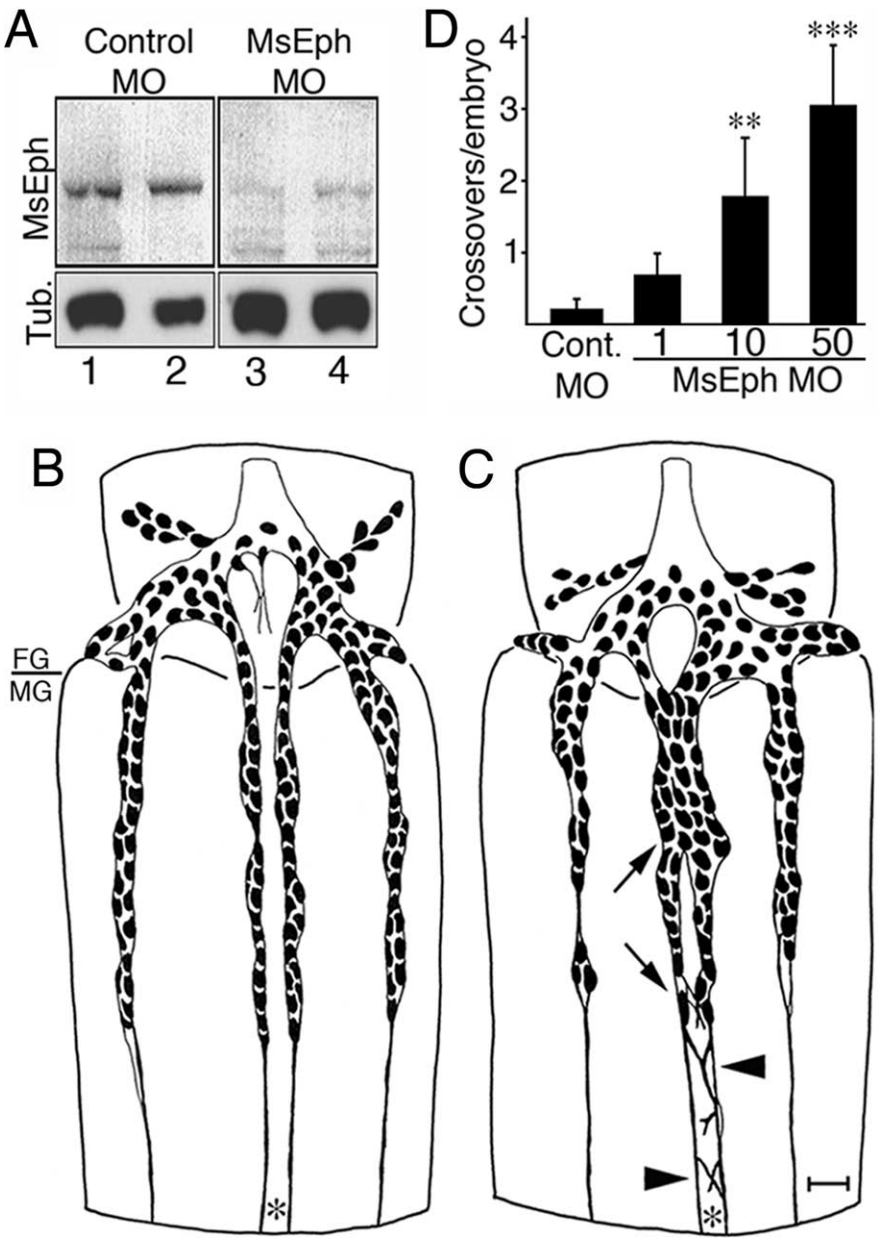


Figure 2. Morpholino treatments that knock down MsEph receptor expression cause midline crossover phenotypes in the developing ENS without hindering normal EP cell migration on the bands. ***A***, ***B***, Effects of morpholino treatments on endogenous MsEph receptor expression in the *Manduca* GV1 cells. ***A***, Immunoblot of lysates from GV1 cells treated with control or MsEph receptor-specific morpholinos (MO; for 48 h); blots were labeled sequentially with antibodies specific for MsEph (top) and tubulin (Tub.; bottom; as a loading control). Preparations treated with MsEph-specific morpholinos showed an average reduction in MsEph expression of 43% (±16% SEM; <0.02), with individual samples ranging from 26 to 57% of control MsEph levels. ***B***, ***C***, Camera lucida images of the developing ENS in morpholino-treated embryo cultures. Asterisks indicate the enteric midline. FG/MG, Foregut–midgut boundary. ***B***, Embryo treated with control morpholinos; the EP cells migrated and grew axons normally along their band pathways (bands not shown) without crossing the enteric midline. ***C***, Embryo treated with MsEph-specific morpholinos; EP cell migration and outgrowth on the bands was normal, but the neurons also exhibited abnormal midline crossovers by both the somata (arrows) and their processes (arrowheads). Scale bar, 25 μm. ***D***, Quantification of the midline crossover phenotypes in morpholino-treated embryos; MsEph receptor-specific morpholinos (MsEph MO) induced a concentration-dependent increase in crossover events (from 1 to 50 μM), whereas control morpholinos (Cont. MO; 50 μM) had no effect. $^{**}p \leq 0.01$; $^{***}p \leq 0.001$.

treatment with MsEph morpholinos led to substantial midline crossing by the EP cells and their processes (Fig. 2*C*), an effect that was strongly concentration dependent (Fig. 2*D*). Because the embryos in these cultures were permitted to develop beyond the transient period of MsEph receptor expression in the midline cells (Fig. 1*G*), we were not able to compare directly the residual levels of MsEph in these specific treatment groups. Nevertheless, these results support the model that reverse signaling from MsEph receptors at the midline via MsEphrin on the EP cells plays an important role in maintaining the neurons on their correct pathways.

MsEphrin and activated Src colocalize in the motile EP cells

Previous studies in cell culture have suggested that SFKs may directly or indirectly mediate reverse signaling by type-A Ephrins (Huai and Drescher, 2001; Davy et al., 2004; Lim et al., 2008), although the presence of multiple SFKs with overlapping functions has complicated an analysis of their individual roles *in vivo* (Gauld and Cambier, 2004; Parsons and Parsons, 2004). In contrast, *Drosophila* expresses only two SFKs with partially overlapping functions. Src42A (Src oncogene at chromosome position 42A), which is the closest ortholog to vertebrate Src, plays a predominant role in the maintenance of adherens junctions in tracheal epithelia (Takahashi et al., 2005; Shindo et al., 2008), whereas Src64B (Src oncogene at chromosome position 64B), a second Src ortholog that is unique to invertebrates (Simon et al., 1985; Kussick and Cooper, 1992; Wu et al., 2008), is specifically required for several aspects of oogenesis (Dodson et al., 1998; O'Reilly et al., 2006). However, both proteins 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 cDNA clones encoding *Manduca* Src42A and Src64B (our unpublished observations). Both *Manduca* Src orthologs have the same predicted structural features found in human Src (supplemental Fig. 2*A*, available at www.jneurosci.org as supplemental material), including N-terminal myristoylation sites and conserved tyrosine residues in the "activation loops" of their catalytic domains that correspond to the autophosphorylation site (Y418) in human c-Src (supplemental Fig. 2*B*, available at www.jneurosci.org as supplemental material) (Boggon and Eck, 2004; Parsons and Parsons, 2004; Roskoski, 2004). In immunoblots of *Manduca* embryo lysates, commercial antibodies against both the inactive form of Src (pan Src) and phosphorylated Src (activated Src, or pSrc) detected similar protein bands at ~60 kDa (Fig. 3*A*), corresponding to the predicted sizes of both *Manduca* Src isoforms. In whole-mount immunostained preparations of staged embryos, pan-Src antibodies labeled all cell types associated with the ENS (Fig. 3*B*), including the migrating neurons (arrows),

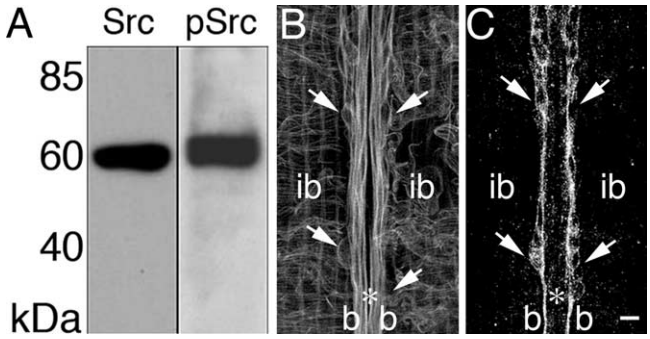


Figure 3. Detection of Src-related proteins in the developing ENS. ***A***, Western blot of *Manduca* embryonic gut tissue using antibodies against inactive (pan) Src and phosphorylated Src (pSrc; anti-pY416). Both antibodies detect an ~60 kDa band (the predicted size of both MsSrc isoforms). ***B***, Anti-pan Src antibodies immunostain all cells associated with the developing ENS, including the EP cells (arrows), their underlying band pathways, adjacent interband muscle, and midline cells. ***C***, Anti-pSrc antibodies produce a much more restricted pattern of immunostaining that is substantially elevated in the EP cells and their growing axons compared with much lower levels in the surrounding musculature. Asterisks indicate the dorsal midline cells. b, Band pathways; ib, interband muscle. Scale bar, 10 μm.

their underlying band pathways, the midline muscle cells (asterisk), and the lateral interband musculature. In contrast, robust pSrc immunoreactivity was primarily confined to the EP cells and their processes (Fig. 3*C*), suggesting a role for Src activation in the control of their behavior. Studies in both *Drosophila* and *Aplysia* have indicated that similar antibodies against pSrc selectively recognize the activated form of Src42A (Src2 in *Aplysia*) (Shindo et al., 2008; Wu et al., 2008), whereas pan-Src antibodies may label both Src isoforms. For the current studies, however, we have simply noted that both antibodies detect *Manduca* Src.

To demonstrate that phosphorylated Src colocalized with MsEphrin in the motile EP cells, we double labeled embryos at 60% of development with anti-MsEphrin and anti-pSrc antibodies. As shown in Figure 4, pSrc immunoreactivity coincided with MsEphrin staining in the somata of the migrating neurons (Fig. 4*A–C*, arrows), their fasciculated axons (Fig. 4*A–C*, arrowheads), and their leading processes (Fig. 4*D–F*). In comparison, only faint levels of pSrc expression could be detected in the underlying muscle bands and surrounding gut musculature, which were also devoid of MsEphrin. At higher magnification (Fig. 4*G–I*), MsEphrin antibodies uniformly labeled the leading processes and filopodia of the EP cells that extended along their band pathways, as reported previously (Coate et al., 2008). In contrast, pSrc immunoreactivity was distributed in a more punctate pattern within their growth cones and filopodia (Fig. 4*G–I*, arrowheads), as well as more sparsely in the underlying muscle cells. Possibly, this punctate pattern of staining reflects the preferential distribution of activated SFKs to membrane microdomains, as reported in other cell types (Navarro et al., 2004; Sverdlov et al., 2007). The colocalization of MsEphrin with activated Src in the leading processes of migratory EP cells suggested that they might functionally interact in mediating reverse signaling responses to MsEph receptors encountered at the midline.

Inhibiting Src in the migrating EP cells causes midline crossover phenotypes

If Src activation is required for MsEphrin reverse signaling, we would predict that inhibiting Src in the EP cells should induce the same types of crossover phenotypes caused by knocking down MsEph receptor expression (Fig. 2). To explore this issue, we first tested whether commercial inhibitors of mammalian SFKs (PP2 and SU6656) could also block Src activation. As shown in supplemental Figure 3 (available at www.jneurosci.org as supplemental material), treating cultured embryos (at 62% of development) with 10 μM PP2 for 1 h resulted in a dramatic reduction in pSrc levels within the EP cells compared with control preparations. Quantification of the relative levels of pSrc immunofluorescence in the EP cells showed that PP2 caused an ~50% reduction in pSrc levels during this period compared with preparations treated with vehicle alone (0.1% DMSO) (supplemental Fig. 3*G*, available at www.jneurosci.org as supplemental material). Treatment with the structurally unrelated inhibitor SU6656 caused a similar reduction in pSrc levels (data not shown), whereas no significant changes were seen in preparations treated with the inactive analog PP3 (discussed below). These results indicated that PP2 and SU6656 were effective at targeting endogenous Src activity within the developing ENS.

To test the effects of Src inhibition on the motile behavior of the EP cells *in vivo*, we opened embryos at 53% of development (just before migration onset) and treated the EP cells with PP2 or SU6656 for 24 h (through the period of migration and axon outgrowth). The preparations were then fixed and immunostained with anti-MsFas II to reveal the full extent of the neurons and their processes in the ENS. Somewhat surprisingly, neither inhibitor caused significant changes in the overall extent of migration and outgrowth along the muscle band pathways, indicating that many aspects of EP cell development had proceeded normally. However, both PP2 (Fig. 5*B*,*E*) and SU6656 (Fig. 5*C*,*F*) caused a dramatic increase in midline crossover events by the neurons compared with controls (Fig. 5*A*,*D*). This aberrant behavior was seen in both the migratory cells (Fig. 5*B*,*C*, arrowheads) and their growing processes (Fig. 5*E*,*F*, arrowheads), in contrast to the precise trajectories normally maintained by the neurons along the muscle bands (Fig. 5*A*,*D*). Quantification of crossover frequencies in the different treatment groups (Fig. 5*J*) showed that PP2 induced midline crossing in a dose-dependent manner (from 1 to 100 μM) that was statistically different from controls at concentrations of $\geq$10 μM. Likewise, treatment with 10 μM SU6656 also caused a significant increase in crossover events ($p > 0.001$) (Fig. 5*J*). In contrast, neither of these inhibitors induced inappropriate migration onto the lateral interband regions, indicating that signaling mechanisms controlling other aspects of EP cell guidance remained intact.

During the first several hours of outgrowth by the EP cells (60–62% of development), their growth cones extend and retract numerous fine filopodia onto both the band pathways and adjacent nonpathway domains (Copenhaver, 1993, 2007). Normally, filopodia that contact the muscle band pathways tend to be incorporated into their leading processes, whereas filopodia extended onto the midline cells (and more lateral interband regions) are rapidly retracted. Using a short-term culture assay to examine the behavior of EP cell filopodia during this period, we found that both PP2 and SU6656 induced a marked increase in the number of filopodia that extended across the midline (Fig. 5*H*,*I*, arrowheads), in contrast to their normal pattern of restricted growth along the bands (Fig. 5*G*). As indicated in Figure 5*K*, this increase in ectopic midline filopodia was highly significant for both Src inhibitors. In contrast, neither compound altered the total number of filopodia (Fig. 5*L*) nor the average length of individual filopodia (Fig. 5*M*) extended by the EP cells on their band pathways compared with controls. These results suggest that the primary role of Src-dependent signaling in the migratory EP cells is to modulate their response to local guidance cues encountered at the enteric midline. This pattern of normal

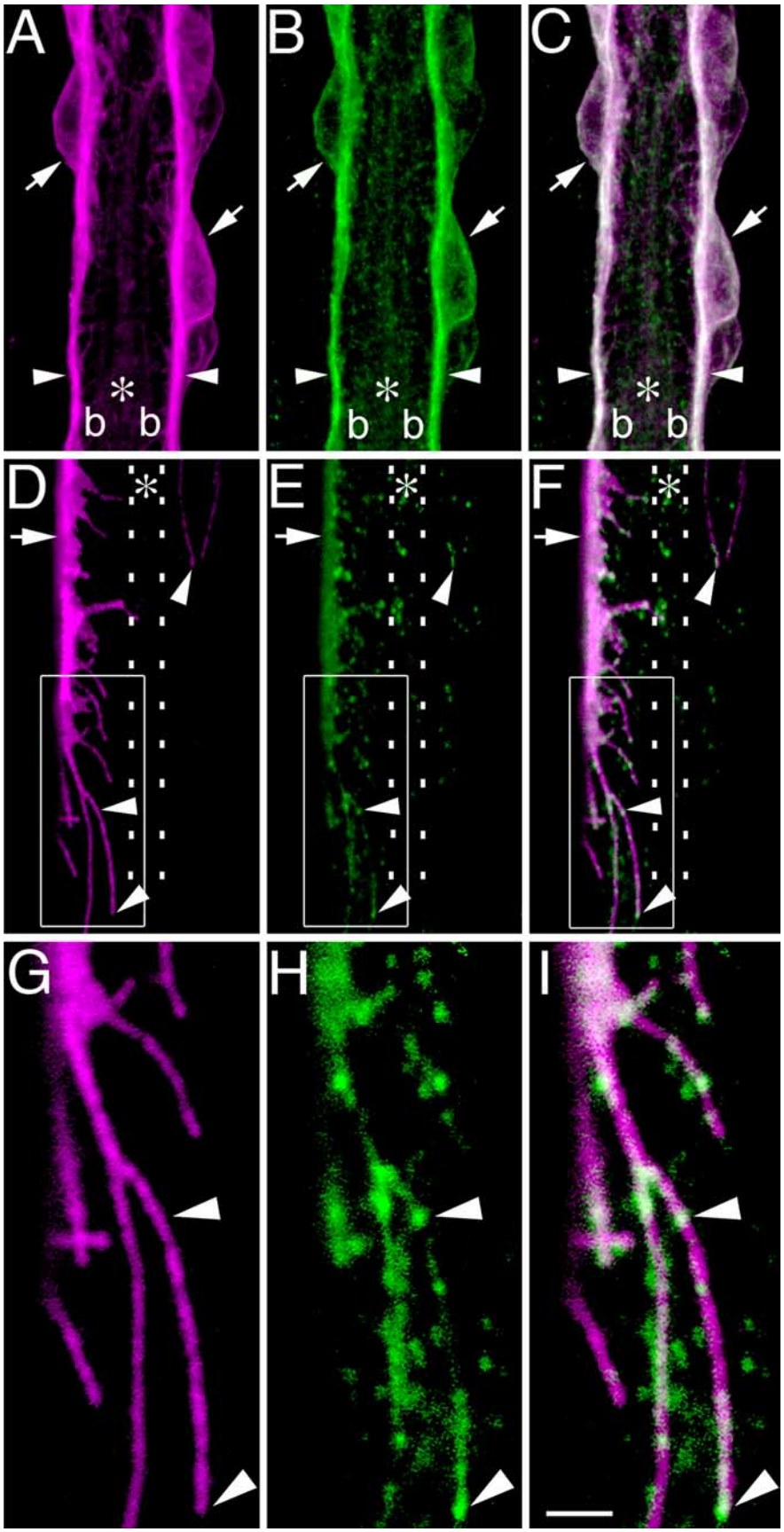


Figure 4. Colocalization of phosphorylated Src and MsEphrin in the EP cells at 65% of development. ***A–C***, Whole-mount immunostaining of the developing ENS with antibodies against MsEphrin (magenta) and phosphorylated Src (green) shows substantial overlap in the EP cell somata (arrows) and axons (arrowheads); lower levels of pSrc immunoreactivity can also be detected in the underlying muscle cells. b, Band pathways. ***D–F***, Colocalization of MsEphrin and pSrc immunoreactivity in their leading processes (arrows) growing posteriorly along the midgut bands (axons on the right dorsal pathway trail those on the left pathway); arrowheads indicate filopodia that have extended in advance of the growth cones along the bands. The dashed white lines show the border of the muscle bands with the midline cells (indicated with asterisks in ***A–F***). ***G–I***, Magnified view of boxed regions in ***D–F***; uniform MsEphrin staining and more punctate pSrc immunostaining is associated with EP cell filopodia (anti-MsEphrin antibodies were applied just before fixation; anti-pSrc antibodies were applied after fixation and permeabilization). Some pSrc immunoreactivity can also be seen in the underlying muscle band cells. Scale bar: ***A–F***, 12 μm; ***G–I***, 4 μm.

growth along the muscle bands but inappropriate midline crossing was remarkably similar to the "gain-of-function" phenotype caused by inhibiting MsEph receptor expression in the ENS (Fig. 2) or by blocking endogenous MsEphrin–MsEph receptor interactions (Coate et al., 2008), suggesting that *Manduca* Src may indeed play a role in modulating MsEphrin-dependent reverse signaling.

Reverse signaling by MsEphrin activates Src within the developing ENS

We previously demonstrated that hyperstimulating reverse signaling in the EP cells with dimeric MsEph-Fc fusion proteins caused a global inhibition of their migration and outgrowth (Coate et al., 2008), supporting the model that MsEph receptors encountered at the enteric midline normally repel the neurons by activating an MsEphrin-dependent signaling pathway (Fig. 1*J*). To test whether reverse signaling by MsEphrin also stimulates the activation of *Manduca* Src, we established an explant assay for analyzing the relative levels of Src phosphorylation in the developing ENS (Fig. 6). Embryonic gut segments containing the ENS were dissected from preparations at 60–62% of development and transferred individually into microcentrifuge tubes containing either dimeric MsEph-Fc (Fig. 6*A*) or a variety of control fusion proteins (including Fc alone and monomeric MsEph-6His). Given our previous studies demonstrating that MsEph-Fc only binds to the MsEphrin-expressing EP cells in the ENS (Coate et al., 2007), this protocol provided a means of testing the effects of MsEph-Fc treatments specifically on the migratory neurons. After incubating the isolated gut cultures for 1–30 min at RT, lysates from each culture were probed in Western blots with anti-pSrc antibodies (one gut per lane). As illustrated in Figure 6*B* (lane 1), treating isolated gut segments with MsEph-Fc led to a dramatic stimulation of Src phosphor-

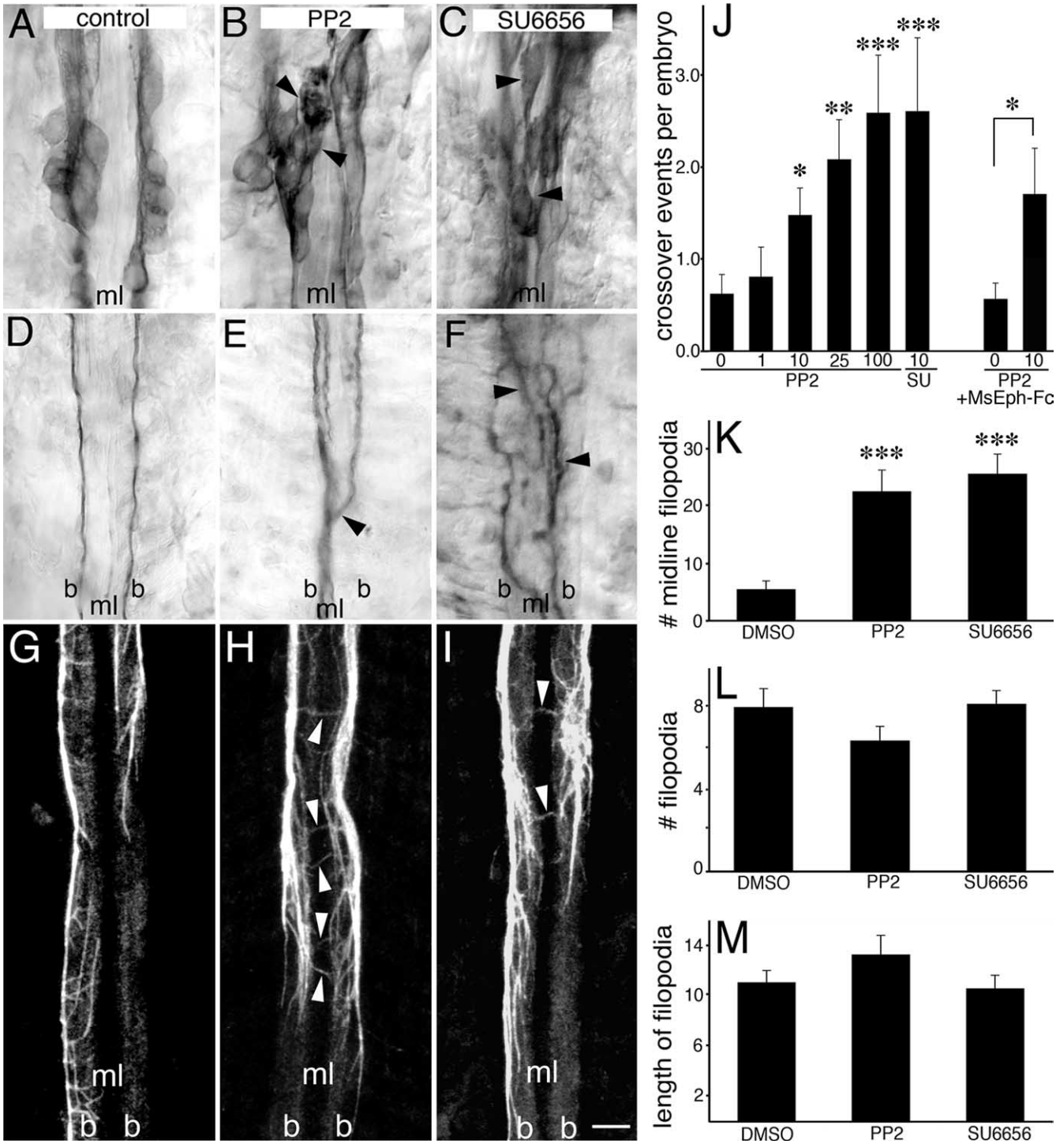


Figure 5. Inhibition of Src activity in the developing ENS induces midline crossover phenotypes. ***A–F***, Embryos were treated at the onset of EP cell migration, allowed to develop for 12 h (through the period of migration and outgrowth), and immunostained with anti-MsFas II antibodies. ***A–C***, The postmigratory EP cells. ***D–F***, Their axonal projections along the midgut. ***A***, ***D***, An embryo that was treated with control medium (plus DMSO); the EP cells exhibited the normal pattern of migration and outgrowth along the band pathways without crossing the midline. ***B***, ***E***, An embryo treated with PP2 exhibited midline crossovers by both the EP cells (***B***, arrowheads) and their axons (***E***, arrowhead), although the overall extent of migration and outgrowth along the band pathways was normal. ***C***, ***F***, An embryo treated with SU6656 exhibited a more severe example of midline crossovers by the EP cells (***C***, arrowheads) and their leading processes (***F***, arrowheads). ***G–I***, Treating the EP cells with Src inhibitors in short-term cultures also caused midline crossing by their growth cones and filopodia (immunostained after 3 h with anti-MsFas II antibodies). ***G***, EP cell filopodia in a DMSO-treated control embryo extended across the muscle band pathways but typically did not grow onto the midline. ***H***, ***I***, In embryos treated with PP2 (***H***) or SU6656 (***I***), a substantial number of filopodia extended across the midline. ***J***, Quantification of the number of crossover events per embryo in the different treatment groups after 24 h in culture. PP2 induced a concentration-dependent increase (from 1 to 100 μM) in the number of midline crossing events compared with controls (including EP cell somata, axons, and filopodia); SU6656 (SU; 10 μM) also caused a significant increase in crossovers. In contrast, the addition of MsEph-Fc alone (20 μg/ml; to stimulate MsEphrin reverse signaling) did not induced the crossover phenotype, whereas adding 20 μg/ml MsEph-Fc plus 10 μM PP2 induced the same frequency of crossovers caused by 10 μM PP2 alone. ***K***, In short-term (3 h) cultures, the average number of EP cell filopodia that grew across the midline was significantly increased in preparations treated with PP2 or SU6656. ***L***, The total number of EP cell filopodia per band pathway was not significantly altered by either Src inhibitor. ***M***, The average length of filopodia was similarly unaffected. Significant differences between groups determined using Student's two-tailed *t* test; $*p \leq 0.05$; $**p \leq 0.01$; $***p \leq 0.001$. Scale bar: ***A–F***, 12 μm; ***G–I***, 5 μm. B, Band pathways; ml, midline.

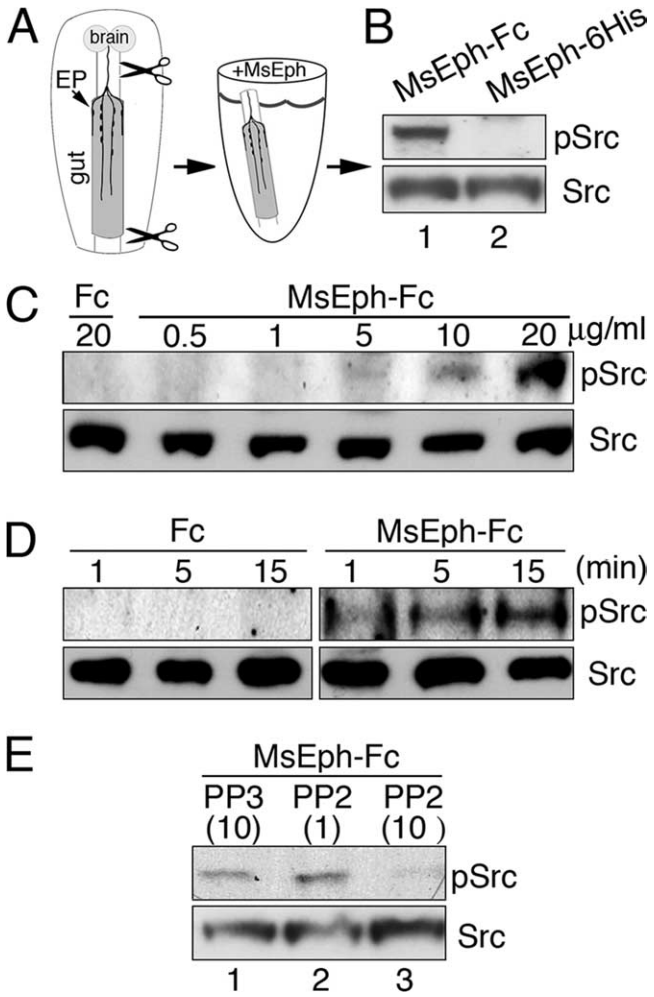


Figure 6. MsEphrin reverse signaling stimulates Src phosphorylation in the ENS of single gut explants. ***A***, Schematic representation of the single gut explant assay; the region of the foregut and midgut encompassing the enteric plexus (including the EP cells and their processes) was removed from embryos at 60–62% of development and transferred individually to Microfuge tubes containing control or MsEph fusion proteins (0.5–20 μg/ml; in 10 μl defined saline). ***B–E***, Western blots probed sequentially with anti-pSrc and anti-Src antibodies; each lane contains the lysate from one embryonic gut explant. ***B***, Incubation with dimeric MsEph-Fc for 15 min (which stimulates MsEphrin reverse signaling in the EP cells) induced a marked increase in the levels of phosphorylated Src (lane 1), whereas monomeric MsEph-6His (which does not stimulate MsEphrin signaling) had no effect (lane 2). ***C***, Single gut explants treated for 15 min with 0.5–20 μg/ml MsEph-Fc showed a concentration-dependent increase in pSrc levels, whereas treatment with control Fc (20 μg/ml) had no effect. ***D***, Treatment with 20 μg/ml MsEph-Fc induced a detectable elevation in pSrc levels in single gut explants within 1 min, and pSrc levels continued to increase over the next 15 min; Fc treatments had no effect during this interval. ***E***, Pretreating gut explants for 20 min with 10 μM PP3 did not prevent the subsequent stimulation of Src phosphorylation by MsEph-Fc (lane 1), nor did pretreatment with 1 μM PP2 (lane 2; a concentration that did not induce midline crossovers; see Fig. 5). In contrast, pretreatment with 10 μM PP2 (which did induce crossovers) almost completely blocked Src phosphorylation in response to MsEph-Fc (lane 3).

ylation above basal levels (which were below the level of detection in this assay). In contrast, no increase in pSrc levels was seen in mock-treated gut cultures (data not shown) or cultures treated with monomeric MsEph-6His (Fig. 6*B*, lane 2), consistent with previous data showing that MsEph-6His can bind MsEphrin but does not stimulate reverse signaling (Coate et al., 2008). Treating gut preparations with a range of concentrations showed that exposure to 0.5–1 μg/ml MsEph-Fc caused no detectable increase in pSrc levels (compared with Fc controls) (Fig. 6*C*), whereas concentrations of $\geq$5 μg/ml induced a concentration-dependent increase in Src phosphorylation. Similarly, when we probed isolated guts with either control Fc or MsEph-Fc (20 μg/ml) for increasing time intervals (Fig. 6*D*), MsEph-Fc induced maximal levels of Src phosphorylation by 15 min, whereas guts treated with control Fc showed no detectable stimulation of pSrc over this period.

To verify the specificity of this effect, we pretreated gut explants with either the Src inhibitor PP2 or its inactive analog PP3 for 20 min. MsEph-Fc was then added to the preparations (20 μg/ml final concentration) for an additional 20 min. As shown in Figure 6*E*, pretreating gut explants with 10 μM PP3 (lane 1) did not prevent the subsequent activation of *Manduca* Src by MsEph-Fc. Likewise, pretreatment with 1 μM PP2 (a concentration that did not induce midline crossovers) (Fig. 5*J*) also did not inhibit the subsequent stimulation of pSrc levels (Fig. 6*C*, lane 2). However, pretreatment with 10 μM PP2 markedly reduced the ability of MsEph-Fc to activate Src activity (Fig. 6*C*, lane 3); this same concentration caused a significant increase in midline crossovers by the EP cells (Fig. 5*B*,*J*). These results indicate that the induction of MsEphrin reverse signaling stimulates *Manduca* Src activity within the developing ENS, consistent with the hypothesis that the endogenous regulation of Src in the EP cells plays an important role in this process.

Next, to test whether MsEphrin reverse signaling specifically stimulates Src phosphorylation in the EP cells, we treated cultured embryos (at 60–62% of development) with either control Fc or MsEph-Fc (20 μg/ml) for 20 min at RT. In addition, we also pretreated matched preparations with either PP2 or PP3, before the addition of MsEph-Fc. (Note that the duration of these experiments was substantially shorter than the incubation periods used to assay the motile behavior of the EP cells) (Fig. 5). We then rapidly fixed the preparations and double immunostained them with anti-pSrc antibodies and anti-MsFas II (as a neuronal marker). Immunofluorescence levels were then analyzed in the growth cones and associated filopodia of the EP cells by confocal microscopy. For each preparation, we captured a 3.5 μm confocal *Z*-stack of the leading filopodia (8 $\times$ 0.5 μm sections), and relative fluorescent intensities were calculated independently for both pSrc and MsFas II immunostaining in the neurons. To optimize our detection of significant changes in Src phosphorylation, we used the anti-pSrc antibody at a dilution of 1:500 (rather than 1:100, as illustrated in Fig. 3 and supplemental Fig. 3, available at www.jneurosci.org as supplemental material); at this concentration, the intensity of pSrc-specific immunofluorescence remained within a linear range (data not shown). Under these conditions, pSrc staining was barely detectable in the growth cones and filopodia of EP cells of untreated preparations or preparations treated with control Fc (Fig. 7*A*,*B*, green). In contrast, treatment with 50 μg/ml MsEph-Fc (to stimulate maximal MsEphrin reverse signaling) induced a marked increase in pSrc staining within the EP cells and their leading processes (Fig. 7*C*,*D*), whereas no change in pSrc levels were apparent in the underlying musculature. Apparent levels of MsFas II in the EP cells also remained unchanged in both treatment groups (Fig. 7, magenta). When visualized at higher magnification, pSrc levels were seen to be substantially elevated within the leading processes and filopodia of the neurons (Fig. 7*G*,*H*), whereas only sparse pSrc immunoreactivity could be detected in the growth cones of matched control preparations (Fig. 7*E*,*F*).

To quantify this effect, we used ImageJ software to measure the relative intensities of pSrc immunoreactivity within the filopodia of each preparation, 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. As shown in Figure 7*I*, no significant changes in MsFas II immunofluorescence were observed in any of the control or treatment groups. In contrast, the relative levels of pSrc immunoreactivity were almost 300% higher in preparations treated with MsEph-Fc compared with preparations treated with control Fc proteins (Fig. 7*J*). Notably, pretreatment with PP2 almost completely abolished this response, whereas pretreatment with PP3 had no effect, consistent with the biochemical data obtained using our single gut explant assay (Fig. 6). These results indicated that stimulating MsEphrin reverse signaling induced a significant increase in Src activation specifically within the EP cells, an effect that could be readily detected in their leading processes and filopodia. In addition, these experiments verified that PP2 effectively blocked the response elicited in these neurons by MsEphrin reverse signaling.

Src activation is required for MsEphrin-dependent reverse signaling in the migratory EP cells

Based on the foregoing results, we postulated that MsEphrin-dependent activation of Src within the EP cells in response to MsEph receptors encountered at the midline normally restricts the neurons from growing onto this inhibitory region (Fig. 8*A*). By this model, treatment with MsEph-Fc (to hyperstimulate reverse signaling) should inhibit EP cell migration and outgrowth in a Src-dependent manner (Fig. 8*B*), whereas blocking Src activation should preclude this response and permit ectopic midline crossing (Fig. 8*C*). We therefore used our embryo culture preparation to test whether the effects of MsEph-Fc treatments on EP cell motility *in vivo* requires Src activation. In embryos treated at the onset of EP cell migration with 0.1% DMSO alone, the neurons migrated and extended axons normally, remaining closely associated with their band pathways while avoiding the midline (Fig. 8*D*, asterisk). In contrast, treating the EP cells with an intermediate concentration of MsEph-Fc (20 μg/ml) caused a significant reduction in the overall extent of migration and outgrowth (Fig. 8*E*); these results are consistent with our previous published studies (Coate et al., 2008). However, when we treated the developing ENS with MsEph-Fc plus 10 μM PP2, the extent of migration and outgrowth was al-

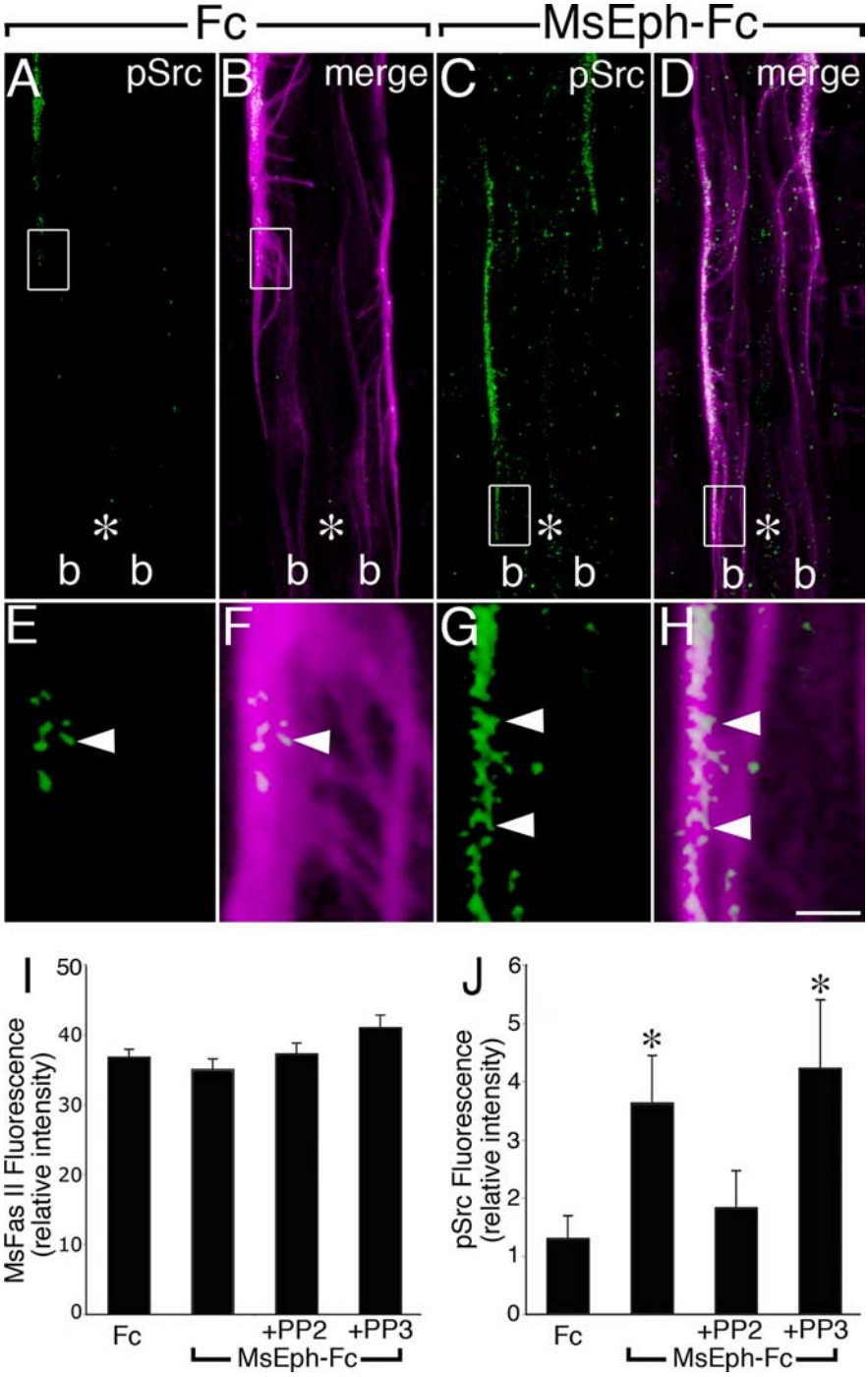


Figure 7. MsEphrin reverse signaling activates endogenous Src within the growth cones of EP cells *in vivo*. ***A–H***, Embryos were treated for 25 min with either control Fc or MsEph-Fc (20 μg/ml) and double immunostained with anti-pSrc antibodies (1:500 dilution; green) plus anti-MsFas II to label the leading processes of the EP cells (magenta). B, Bands; asterisks, midline cells. ***A***, ***B***, A preparation treated with control Fc exhibited only low levels of detectable pSrc in the growth cones of the EP cells (immunostained with anti-pSrc at 1:500 dilution; see Materials and Methods). ***C***, ***D***, A preparation treated with MsEph-Fc exhibited a marked enrichment of pSrc in EP cell growth cones and leading filopodia but not in the underlying bands or midline cells, consistent with previous evidence that MsEph-Fc stimulates MsEphrin reverse signaling specifically in the EP cells. ***E***, ***F***, Higher magnification of the boxed regions in ***A*** and ***B***, revealing sparse levels of pSrc activity in the filopodia of EP cells treated with control Fc. ***G***, ***H***, Higher magnification of the boxed regions in ***C*** and ***D***, showing enhanced levels of pSrc immunoreactivity in growth cones treated with MsEph-Fc. ***I***, The relative levels of MsFas II-specific immunofluorescence in the EP cell processes were unaffected by treatment with Fc, MsEph-Fc, PP2, or PP3. ***J***, Relative levels of pSrc-specific immunofluorescence were significantly increased by treatment with MsEph-Fc compared with controls; this increase was inhibited by pretreatment with PP2 but not PP3 (10 μM). Fluorescent intensities for the two fluorochromes were independently measured and normalized to background levels in each preparation (Coate et al., 2008). *$p < 0.05$. Scale bar: ***A–D***, 10 μM; ***E–H***, 2.5 μM.

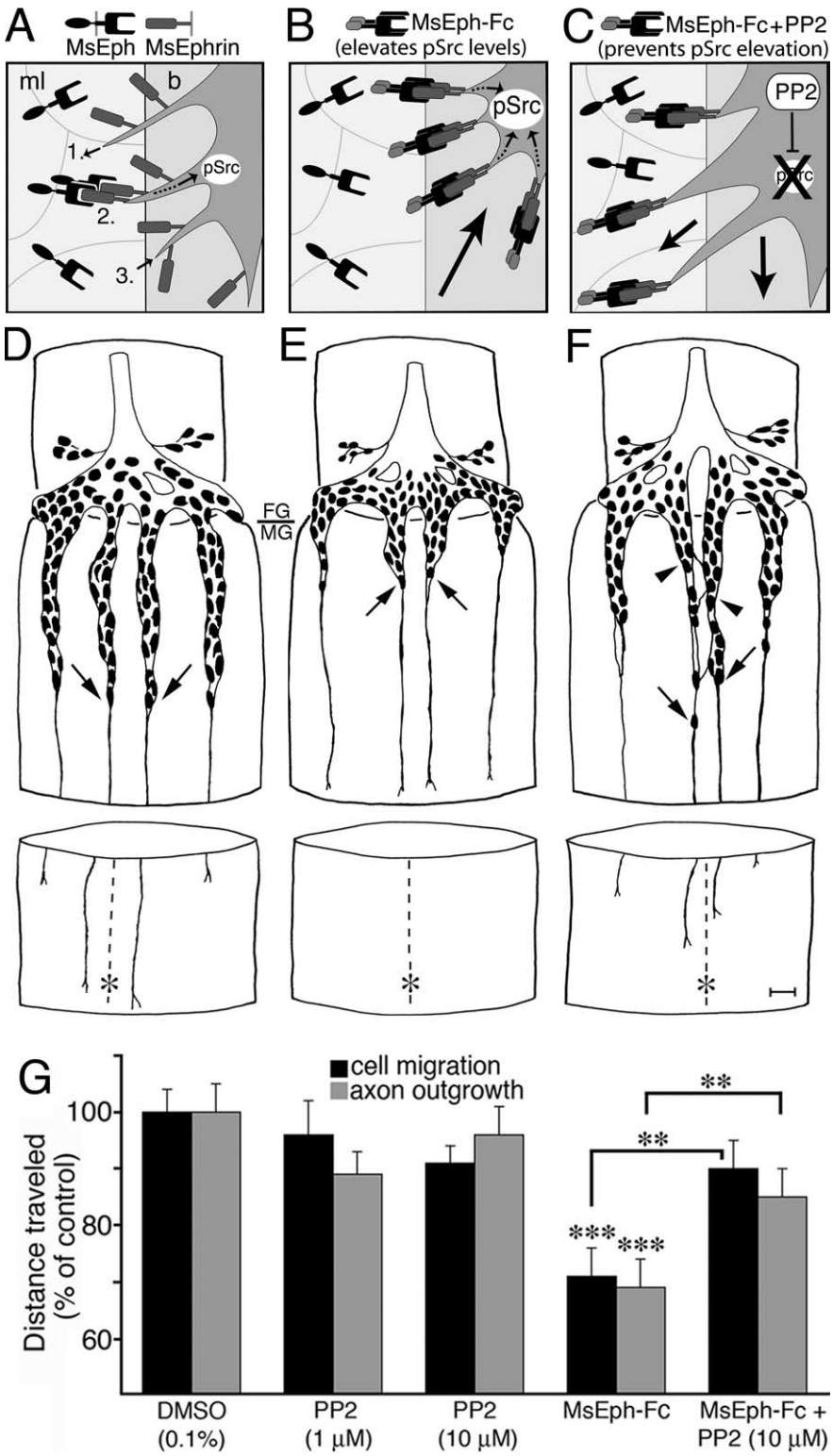


Figure 8. Src activation is required for MsEphrin-mediated reverse signaling in the migratory EP cells. ***A–C***, Model for Src-dependent reverse signaling by MsEphrin. ***A***, During normal development, MsEph receptors at the midline (ml) stimulate MsEphrin-dependent Src activation in the EP cells, leading to local filopodial retraction. ***B***, Overstimulating this reverse signaling response with MsEph-Fc hyperactivates Src in the EP cells, resulting in a general inhibition of EP cell motility. ***C***, Pretreatment with the Src inhibitor PP2 should therefore prevent the effects of exogenous MsEph-Fc and might also interfere with reverse signaling by endogenous MsEph receptors at the midline, leading to midline crossovers. ***D–F***, Camera lucida images of the ENS in cultured embryos immunostained with MsFas II antibodies. Arrows indicate the most distal EP cells on the dorsal band pathways; asterisks mark the enteric midline; the gap between upper and lower gut segments is 300 μm. ***D***, In embryos treated with control culture medium (plus DMSO), the EP cells exhibited normal migration and outgrowth along their band pathways without crossing the midline. ***E***, In embryos treated with an intermediate concentration of MsEph-Fc (20 μg/ml), EP cell migration and outgrowth along the bands was partially inhibited. ***F***, In embryos treated with both MsEph-Fc and PP2, the normal extent of EP cell migration most fully restored (Fig. 8*F*,*G*). In addition, PP2 plus MsEph-Fc induced the same frequency of midline crossing events seen in the absence of MsEph-Fc (Fig. 5*J*), whereas PP2 alone had no significant effect on the overall extent of migration and outgrowth (Fig. 8*G*). Thus, we were unable to induce the reverse signaling response when Src activity was blocked. In combination with our evidence that dimeric MsEph-Fc also stimulates Src phosphorylation in the EP cells (Figs. 6, 7), these results indicate that reverse signaling by a specific GPI-linked Ephrin (MsEphrin) is mediated via the local activation of endogenous Src activity in migratory neurons within the developing ENS.

Discussion

A role for Src-dependent reverse signaling by MsEphrin at the enteric midline

Previously, we showed that reverse signaling by MsEphrin normally prevents the EP cells from crossing the enteric midline: blocking endogenous MsEphrin–MsEph receptor interactions allows the neurons to grow inappropriately across the midline, whereas hyperactivating this response causes a general inhibition of their migration and outgrowth, akin to a collapse-like phenotype (Coate et al., 2008). We have now shown that inhibiting MsEph receptor expression in the midline cells induces the same crossover phenotypes (Fig. 2), as does knocking down MsEphrin expression in the EP cells (Coate et al., 2008). Although MsEphrin can also stimulate conventional forward signaling via MsEph receptors in cell culture (Kaneko and Nighorn, 2003), forward signaling does not appear to play an essential role in the developing ENS. Our experiments in *Manduca* provide the first clear example of reverse signaling by one specific GPI-linked Ephrin in the control of neuronal guidance *in vivo*.

Several different lines of evidence indicate that MsEphrin-dependent reverse signaling is mediated via *Manduca* Src. Stim-

←

and outgrowth along the bands was restored, and the neurons also exhibited an increase in ectopic midline crossovers (arrowheads). Scale bar in ***F***, 25 μm. ***G***, Quantification of the extent of EP cell migration (black histograms) and axon outgrowth (gray histograms) along the midgut bands (normalized to matched sets of controls). PP2 alone did not impede normal EP cell motility, even at concentrations that induced midline crossovers (see Fig. 5). MsEph-Fc (20 μg/ml) caused a significant inhibition of EP cell migration and outgrowth along the bands, an effect that was reversed by PP2. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

ulating MsEphrin reverse signaling with MsEph-Fc caused the rapid elevation of Src activity in the EP cells and inhibited their motile behavior, whereas both effects were blocked by Src inhibitors (Figs. 6–8). Conversely, inhibiting Src activity induced the same midline crossover phenotypes caused by blocking MsEphrin reverse signaling, regardless of whether MsEph-Fc was also introduced (Fig. 5*J*). These results support the model that MsEph receptors at the enteric midline normally restrict the EP cells via MsEphrin-dependent activation of endogenous Src, thereby preventing the neurons from growing onto these inappropriate regions (Fig. 8). Given previous studies showing that SFKs can also promote filopodial outgrowth and stability (Suter et al., 2004; Robles et al., 2005; Wu et al., 2008), it was somewhat surprising that other aspects of EP motility were not altered by the Src inhibitors (Fig. 4). Additional guidance cues encountered by the neurons *in vivo* may help maintain their growth and migration along the band pathways, even when Src signaling is perturbed (Wright et al., 1999; Wright and Copenhaver, 2000).

It is also possible that the two Src isoforms expressed in this system serve opposing functions in the EP cells. As noted above, *Manduca* possesses homologs of both *Drosophila* Src42A (structurally similar to mammalian Src) and Src64B (a more distantly related SFK). In flies, both Src proteins are coexpressed in many tissues but may play distinct roles in controlling cellular interactions (Thomas and Wieschaus, 2004; Takahashi et al., 2005; Shindo et al., 2008). Likewise, recent studies using *Aplysia* neurons have shown that both Src1 (the Molluscan ortholog of Src64B) and Src2 (orthologous to Src42A) are enriched in growth cones but traffic to distinct intracellular compartments, suggesting that they may mediate responses to different environmental stimuli (Wu et al., 2008). Intriguingly, only the phosphorylated forms of Src42A/Src2 but not Src64B/Src1 appear to be recognized by commercial antibodies against pSrc (Shindo et al., 2008; Wu et al., 2008), suggesting that the dramatic enhancement of pSrc staining in the EP cells specifically reflects the activation of Src42A (Figs. 3, 4; and supplemental Fig. 3, available at www.jneurosci.org as supplemental material). Similarly, the ability of Src inhibitors to promote ectopic midline crossovers and prevent MsEphrin reverse signaling may be attributable to their specific effects on Src42A, although this possibility remains to be verified.

Induction of a repulsive response by a GPI-linked Ephrin

Reverse signaling via Ephrin-B ligands can either stimulate or inhibit neuronal motility via the modulation of several different signaling pathways, depending on the cellular context (Wilkinson, 2001; Kullander and Klein, 2002; Pasquale, 2005). In contrast, the mechanisms associated with Ephrin-A reverse signaling have remained poorly understood. Previous work in cell culture showed that Ephrin-A reverse signaling enhanced the concentration of phosphorylated Fyn in lipid microdomains without stimulating Fyn phosphorylation per se (Davy et al., 1999; Davy and Robbins, 2000). Conversely, another study reported that treatment with Eph-Fc constructs altered the phosphorylation of several proteins but not Fyn or FAK (Huai and Drescher, 2001). Intriguingly, Ephrin-A reverse signaling was recently shown to activate SFKs and inhibit apoptotic cell death in T-cells and T-cell lymphomas, suggesting that this pathway may also serve important functions in a variety of diseases (Holen et al., 2008). In our current work, we showed that MsEphrin reverse signaling led to the rapid stimulation of Src activity in the EP cells (within 1 min) (Figs. 6, 7), providing new evidence that endogenous SFKs may transduce their response to EphA receptors encountered at the midline. Moreover, given our demonstration that MsEph-Fc dimers are fully capable of stimulating a reverse signaling response through GPI-linked Ephrins, the widespread use of EphA-Fc constructs to block Ephrin-A forward signaling may require reevaluation (Brantley et al., 2002; Chadaram et al., 2007; Passante et al., 2008).

Previous studies *in vitro* have indicated that reverse signaling via Ephrin-A ligands can enhance cell adhesion and outgrowth (Davy and Robbins, 2000; Huai and Drescher, 2001; Knoll and Drescher, 2002). Likewise, Ephrin-A reverse signaling most likely promotes the formation of appropriate neuronal projections within the mouse vomeronasal system (Knoll et al., 2001a,b; Knoll and Drescher, 2002) and may support the outgrowth of spinal motor neurons (Eberhart et al., 2004; Marquardt et al., 2005). In contrast, we have shown that MsEphrin reverse signaling induces a repulsive response in the EP cells (Fig. 8), substantiating the model that MsEphrin-MsEph receptor interactions restrict the guidance of the migratory neurons at the enteric midline. Recent work on retinocollicular development has also suggested that reverse signaling by Ephrin-A ligands may serve a repellant function in the establishment of anterior-posterior topographic projections (Rashid et al., 2005; Lim et al., 2008).

How might signaling via a GPI-linked protein like MsEphrin induce the activation of nonreceptor tyrosine kinases? By analogy to other GPI-linked receptors (Zisch et al., 1995; Airaksinen et al., 2006), Ephrin-A ligands may interact with transmembrane coreceptors capable of modulating SFK recruitment and activity. For example, Lim et al. (2008) recently showed that the p75 neurotrophin receptor may be required for Ephrin-A reverse signaling in retinal axons, although other transmembrane proteins must serve this function in non-neuronal cells (and invertebrate systems) that express GPI-linked Ephrins but lack p75 (Bothwell, 2006; Underwood and Coulson, 2008). An alternative model is that activated Ephrins may cluster into lipid microdomains, creating a microenvironment that favors the autoactivation of SFKs (Ogawa et al., 2000; Gauthier and Robbins, 2003; Murai and Pasquale, 2003). Numerous studies have shown that both GPI-linked membrane proteins (Chatterjee and Mayor, 2001; Fujita and Jigami, 2008) and myristoylated/palmitoylated SFKs (Resh, 1994; Mukherjee et al., 2003) partition to sphingolipid-rich membrane compartments, supporting the model that Ephrin-A signaling occurs in the context of lipid "rafts" (Simons and Toomre, 2000). Indeed, Ephrin-A ligands can be readily partitioned to lipid raft fractions by biochemical methods (Ogawa et al., 2000; Huai and Drescher, 2001; Gauthier and Robbins, 2003), whereas punctate patterns of Ephrin-A immunostaining have been hypothesized to represent local concentrations within specific membrane compartments (Davy et al., 1999; Harbott and Nobes, 2005; Lim et al., 2008).

In contrast, we consistently found that MsEphrin appears to be uniformly distributed throughout the plasma membrane of the EP cells and their processes (Figs. 1, 4). Of note is that we routinely applied MsEphrin antibodies to the developing ENS just before fixation, which may have helped minimize histochemical artifacts associated with detergent-based methods for permeabilization (Heffer-Lauc et al., 2007). Labeling unfixed preparations with MsEph-Fc revealed a similar uniform distribution of bioavailable MsEphrin ligands on the EP cells (Coate et al., 2007). These results suggest that the localization of MsEphrin to membrane microdomains within living neurons is well below the level of resolution obtained by confocal imaging. In comparison, anti-pSrc staining (which could only be visualized in fixed, permeabilized preparations) was concentrated in punctate regions within the EP cells (Figs. 4, 7), suggesting that MsEphrin-dependent

reverse signaling may be selectively transduced within membrane microdomains. However, given recent evidence indicating that this type of punctate pSrc immunostaining may also be the result of detergent extraction (Wu et al., 2008), the physiological relevance of this observation remains to be explored.

Although the most parsimonious explanation for our results is that overstimulating MsEphrin reverse signaling in the EP cells (with MsEph-Fc) caused the hyperactivation of a normal repulsive response, an alternative interpretation is that dimeric MsEph-Fc can induce a Src-dependent adhesive interaction with other guidance cues, which in turn impedes neuronal growth. For example, studies in cell culture have suggested that Ephrin-A reverse signaling may promote adhesion via the "inside-out" activation of integrins (Davy and Robbins, 2000; Huai and Drescher, 2001; Knoll and Drescher, 2002). However, this model would predict that blocking Src activity in the EP cells would enhance their motile behavior, whereas neither PP2 nor SU6656 altered their normal growth along the band pathways. Whether MsEphrin-dependent signaling also modulates the effects of other guidance receptors to promote an integrated migratory response within the developing ENS remains to be explored.

References

Airaksinen MS, Holm L, Hatinen T (2006) Evolution of the GDNF family ligands and receptors. Brain Behav Evol 68:181–190.

Alland L, Peseckis SM, Atherton RE, Berthiaume L, Resh MD (1994) Dual myristylation and palmitylation of Src family member p59fyn affects subcellular localization. J Biol Chem 269:16701–16705.

Boggon TJ, Eck MJ (2004) Structure and regulation of Src family kinases. Oncogene 23:7918–7927.

Bothwell M (2006) Evolution of the neurotrophin signaling system in invertebrates. Brain Behav Evol 68:124–132.

Brantley DM, Cheng N, Thompson EJ, Lin Q, Brekken RA, Thorpe PE, Muraoka RS, Cerretti DP, Pozzi A, Jackson D, Lin C, Chen J (2002) Soluble Eph A receptors inhibit tumor angiogenesis and progression in vivo. Oncogene 21:7011–7026.

Brownlee H, Gao PP, Frisen J, Dreyfus C, Zhou R, Black IB (2000) Multiple ephrins regulate hippocampal neurite outgrowth. J Comp Neurol 425:315–322.

Chadaram SR, Laskowski MB, Madison RD (2007) Topographic specificity within membranes of a single muscle detected *in vitro*. J Neurosci 27:13938–13948.

Chatterjee S, Mayor S (2001) The GPI-anchor and protein sorting. Cell Mol Life Sci 58:1969–1987.

Chen CL, Lampe DJ, Robertson HM, Nardi JB (1997) Neuroglian is expressed on cells destined to form the prothoracic glands of *Manduca* embryos as they segregate from surrounding cells and rearrange during morphogenesis. Dev Biol 181:1–13.

Coate TM, Swanson TL, Proctor TM, Nighorn AJ, Copenhaver PF (2007) Eph receptor expression defines midline boundaries for ephrin-positive migratory neurons in the enteric nervous system of *Manduca sexta*. J Comp Neurol 502:175–191.

Coate TM, Wirz JA, Copenhaver PF (2008) Reverse signaling via glycosylphosphatidylinositol-linked Ephrin prevents midline crossing by migratory neurons during embryonic development in *Manduca*. J Neurosci 28:3846–3860.

Copenhaver PF (1993) Origins, migration, and differentiation of glial cells in the insect nervous system from a discrete set of glial precursors. Development 117:59–74.

Copenhaver PF (2007) How to innervate a simple gut: familiar themes and unique aspects in the formation of the insect enteric nervous system. Dev Dyn 236:1841–1864.

Copenhaver PF, Taghert PH (1989a) Development of the enteric nervous system in the moth I. Diversity of cell types and the embryonic expression of FMRFamide-related neuropeptides. Dev Biol 131:70–84.

Copenhaver PF, Taghert PH (1989b) Development of the enteric nervous system in the moth II. Stereotyped cell migration precedes the differentiation of embryonic neurons. Dev Biol 131:85–101.

Copenhaver PF, Taghert PH (1990) Neurogenesis in the insect enteric nervous system: generation of pre-migratory neurons from an epithelial placode. Development 109:17–28.

Dalva MB, Takasu MA, Lin MZ, Shamah SM, Hu L, Gale NW, Greenberg ME (2000) EphB receptors interact with NMDA receptors and regulate excitatory synapse formation. Cell 103:945–956.

Davy A, Robbins SM (2000) Ephrin-A5 modulates cell adhesion and morphology in an integrin-dependent manner. EMBO J 19:5396–5405.

Davy A, Soriano P (2005) Ephrin signaling in vivo: look both ways. Dev Dyn 232:1–10.

Davy A, Gale NW, Murray EW, Klinghoffer RA, Soriano P, Feuerstein C, Robbins SM (1999) Compartmentalized signaling by GPI-anchored ephrin-A5 requires the Fyn tyrosine kinase to regulate cellular adhesion. Genes Dev 13:3125–3135.

Davy A, Aubin J, Soriano P (2004) Ephrin-B1 forward and reverse signaling are required during mouse development. Genes Dev 18:572–583.

Dodson GS, Guarnieri DJ, Simon MA (1998) Src64 is required for ovarian ring canal morphogenesis during *Drosophila* oogenesis. Development 125:2883–2892.

Eberhart J, Barr J, O'Connell S, Flagg A, Swartz ME, Cramer KS, Tosney KW, Pasquale EB, Krull CE (2004) Ephrin-A5 exerts positive or inhibitory effects on distinct subsets of EphA4-positive motor neurons. J Neurosci 24:1070–1078.

Egea J, Klein R (2007) Bidirectional Eph-ephrin signaling during axon guidance. Trends Cell Biol 17:230–238.

Egea J, Nissen UV, Dufour A, Sahin M, Greer P, Kullander K, Mrsic-Flogel TD, Greenberg ME, Kiehn O, Vanderhaeghen P, Klein R (2005) Regulation of EphA 4 kinase activity is required for a subset of axon guidance decisions suggesting a key role for receptor clustering in Eph function. Neuron 47:515–528.

Fujita M, Jigami Y (2008) Lipid remodeling of GPI-anchored proteins and its function. Biochim Biophys Acta 1780:410–420.

Garcia MG, Toney SJ, Hille MB (2004) Focal adhesion kinase (FAK) expression and phosphorylation in sea urchin embryos. Gene Expr Patterns 4:223–234.

Gauld SB, Cambier JC (2004) Src-family kinases in B-cell development and signaling. Oncogene 23:8001–8006.

Gauthier LR, Robbins SM (2003) Ephrin signaling: One raft to rule them all? One raft to sort them? One raft to spread their call and in signaling bind them? Life Sci 74:207–216.

Harbott LK, Nobes CD (2005) A key role for Abl family kinases in EphA receptor-mediated growth cone collapse. Mol Cell Neurosci 30:1–11.

Heffer-Lauc M, Viljetic B, Vajn K, Schnaar RL, Lauc G (2007) Effects of detergents on the redistribution of gangliosides and GPI-anchored proteins in brain tissue sections. J Histochem Cytochem 55:805–812.

Himanen JP, Chumley MJ, Lackmann M, Li C, Barton WA, Jeffrey PD, Vearing C, Geleick D, Feldheim DA, Boyd AW, Henkemeyer M, Nikolov DB (2004) Repelling class discrimination: ephrin-A5 binds to and activates EphB2 receptor signaling. Nat Neurosci 7:501–509.

Hiruma K, Riddiford LM (2004) Differential control of MHR3 promoter activity by isoforms of the ecdysone receptor and inhibitory effects of E75A and MHR3. Dev Biol 272:510–521.

Holen HL, Shadidi M, Narvhus K, Kjosnes O, Tierens A, Aasheim HC (2008) Signaling through ephrin-A ligand leads to activation of Src-family kinases, Akt phosphorylation, and inhibition of antigen receptor-induced apoptosis. J Leukoc Biol 84:1183–1191.

Holmberg J, Armulik A, Senti KA, Edoff K, Spalding K, Momma S, Cassidy R, Flanagan JG, Frisen J (2005) Ephrin-A2 reverse signaling negatively regulates neural progenitor proliferation and neurogenesis. Genes Dev 19:462–471.

Horgan AM, Copenhaver PF (1998) G-protein-mediated inhibition of neuronal migration requires calcium influx. J Neurosci 18:4189–4200.

Horgan AM, Lagrange MT, Copenhaver PF (1995) A developmental role for the heterotrimeric G protein Go_{α} in a migratory population of embryonic neurons. Dev Biol 172:640–653.

Huai J, Drescher U (2001) An ephrin-A-dependent signaling pathway controls integrin function and is linked to the tyrosine phosphorylation of a 120-kDa protein. J Biol Chem 276:6689–6694.

Kaneko M, Nighorn A (2003) Interaxonal Eph-ephrin signaling may mediate sorting of olfactory sensory axons in *Manduca sexta*. J Neurosci 23:11523–11538.

Knoll B, Drescher U (2002) Ephrin-As as receptors in topographic projections. Trends Neurosci 25:145–149.

Knoll B, Zarbalis K, Wurst W, Drescher U (2001a) A role for the EphA family in the topographic targeting of vomeronasal axons. Development 128:895–906.

Knoll B, Isenmann S, Kilic E, Walkenhorst J, Engel S, Wehinger J, Bahr M, Drescher U (2001b) Graded expression patterns of ephrin-As in the superior colliculus after lesion of the adult mouse optic nerve. Mech Dev 106:119–127.

Kullander K, Klein R (2002) Mechanisms and functions of Eph and ephrin signalling. Nat Rev Mol Cell Biol 3:475–486.

Kussick SJ, Cooper JA (1992) Overexpressed *Drosophila* src 64B is phosphorylated at its carboxy-terminal tyrosine, but is not catalytically repressed, in cultured *Drosophila* cells. Oncogene 7:2461–2470.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.

Lan Q, Hiruma K, Hu X, Jindra M, Riddiford LM (1999) Activation of a delayed-early gene encoding MHR3 by the ecdysone receptor heterodimer EcR-B1-USP-1 but not by EcR-B1-USP-2. Mol Cell Biol 19:4897–4906.

Lim YS, McLaughlin T, Sung TC, Santiago A, Lee KF, O'Leary DD (2008) p75(NTR) mediates ephrin-A reverse signaling required for axon repulsion and mapping. Neuron 59:746–758.

Marquardt T, Shirasaki R, Ghosh S, Andrews SE, Carter N, Hunter T, Pfaff SL (2005) Coexpressed EphA receptors and ephrin-A ligands mediate opposing actions on growth cone navigation from distinct membrane domains. Cell 121:127–139.

Mendes SW, Henkemeyer M, Liebl DJ (2006) Multiple Eph receptors and B-class ephrins regulate midline crossing of corpus callosum fibers in the developing mouse forebrain. J Neurosci 26:882–892.

Mukherjee A, Arnaud L, Cooper JA (2003) Lipid-dependent recruitment of neuronal Src to lipid rafts in the brain. J Biol Chem 278:40806–40814.

Murai KK, Pasquale EB (2003) 'Eph'ective signaling: forward, reverse and crosstalk. J Cell Sci 116:2823–2832.

Navarro A, Anand-Apte B, Parat MO (2004) A role for caveolae in cell migration. FASEB J 18:1801–1811.

Ogawa K, Pasqualini R, Lindberg RA, Kain R, Freeman AL, Pasquale EB (2000) The ephrin-A1 ligand and its receptor, EphA2, are expressed during tumor neovascularization. Oncogene 19:6043–6052.

O'Reilly AM, Ballew AC, Miyazawa B, Stocker H, Hafen E, Simon MA (2006) Csk differentially regulates Src64 during distinct morphological events in *Drosophila* germ cells. Development 133:2627–2638.

Parsons SJ, Parsons JT (2004) Src family kinases, key regulators of signal transduction. Oncogene 23:7906–7909.

Pasquale EB (2004) Eph-ephrin promiscuity is now crystal clear. Nat Neurosci 7:417–418.

Pasquale EB (2005) Eph receptor signalling casts a wide net on cell behaviour. Nat Rev Mol Cell Biol 6:462–475.

Passante L, Gaspard N, Degraeve M, Frisen J, Kullander K, De Maertelaer V, Vanderhaeghen P (2008) Temporal regulation of ephrin/Eph signalling is required for the spatial patterning of the mammalian striatum. Development 135:3281–3290.

Rashid T, Upton AL, Blentic A, Ciossek T, Knoll B, Thompson ID, Drescher U (2005) Opposing gradients of ephrin-As and EphA7 in the superior colliculus are essential for topographic mapping in the mammalian visual system. Neuron 47:57–69.

Resh MD (1994) Myristylation and palmitylation of Src family members: the fats of the matter. Cell 76:411–413.

Robles E, Woo S, Gomez TM (2005) Src-dependent tyrosine phosphorylation at the tips of growth cone filopodia promotes extension. J Neurosci 25:7669–7681.

Roskoski R, Jr (2004) Src protein-tyrosine kinase structure and regulation. Biochem Biophys Res Commun 324:1155–1164.

Shindo M, Wada H, Kaido M, Tateno M, Aigaki T, Tsuda L, Hayashi S (2008) Dual function of Src in the maintenance of adherens junctions during tracheal epithelial morphogenesis. Development 135:1355–1364.

Simon MA, Drees B, Kornberg T, Bishop JM (1985) The nucleotide sequence and the tissue-specific expression of *Drosophila* c-src. Cell 42:831–840.

Simons K, Toomre D (2000) Lipid rafts and signal transduction. Nat Rev Mol Cell Biol 1:31–39.

Summerton JE (2005) Endo-Porter: a novel reagent for safe, effective delivery of substances into cells. Ann N Y Acad Sci 1058:62–75.

Suter DM, Schaefer AW, Forscher P (2004) Microtubule dynamics are necessary for SRC family kinase-dependent growth cone steering. Curr Biol 14:1194–1199.

Sverdlov M, Shajahan AN, Minshall RD (2007) Tyrosine phosphorylation-dependence of caveolae-mediated endocytosis. J Cell Mol Med 11:1239–1250.

Swanson TL, Knittel LM, Coate TM, Farley SM, Snyder MA, Copenhaver PF (2005) The insect amyloid precursor protein interacts with the heterotrimeric G protein Goα in an identified population of migratory neurons. Dev Biol 288:160–178.

Takahashi M, Takahashi F, Ui-Tei K, Kojima T, Saigo K (2005) Requirements of genetic interactions between Src42A, armadillo and shotgun, a gene encoding E-cadherin, for normal development in *Drosophila*. Development 132:2547–2559.

Thomas JH, Wieschaus E (2004) src64 and tec29 are required for microfilament contraction during *Drosophila* cellularization. Development 131:863–871.

Thomas SM, Brugge JS (1997) Cellular functions regulated by Src family kinases. Annu Rev Cell Dev Biol 13:513–609.

Underwood CK, Coulson EJ (2008) The p75 neurotrophin receptor. Int J Biochem Cell Biol 40:1664–1668.

Wang X, Roy PJ, Holland SJ, Zhang LW, Culotti JG, Pawson T (1999) Multiple ephrins control cell organization in *C. elegans* using kinase-dependent and -independent functions of the VAB-1 Eph receptor. Mol Cell 4:903–913.

Wilkinson DG (2001) Multiple roles of EPH receptors and ephrins in neural development. Nat Rev Neurosci 2:155–164.

Wright JW, Copenhaver PF (2000) Different isoforms of fasciclin II play distinct roles in the guidance of neuronal migration during insect embryogenesis. Dev Biol 225:59–78.

Wright JW, Snyder MA, Schwinof KM, Combes S, Copenhaver PF (1999) A role for fasciclin II in the guidance of neuronal migration. Development 126:3217–3228.

Wu B, Decourt B, Zabidi MA, Wuethrich LT, Kim WH, Zhou Z, Macisaac K, Suter DM (2008) Microtubule-mediated Src tyrosine kinase trafficking in neuronal growth cones. Mol Biol Cell 19:4611–4627.

Zisch AH, D'Alessandri L, Amrein K, Ranscht B, Winterhalter KH, Vaughn L (1995) The glypiated neuronal cell adhesion molecule contactin/F11 complexes with src-family protein tyrosine kinase Fyn. Mol Cell Neurosci 6:263–279.