Molecules and Cells

Minireview

Molecular Mechanisms Underlying Motor Axon Guidance in *Drosophila*

Sangyun Jeong*

Division of Life Sciences (Molecular Biology Major), Department of Bioactive Material Sciences, and Research Center of Bioactive Materials, Jeonbuk National University, Jeonju 54896, Korea
*Correspondence: sjeong4@jbnu.ac.kr
https://doi.org/10.14348/molcells.2021.0129
www.molcells.org

Decoding the molecular mechanisms underlying axon guidance is key to precise understanding of how complex neural circuits form during neural development. Although substantial progress has been made over the last three decades in identifying numerous axon guidance molecules and their functional roles, little is known about how these guidance molecules collaborate to steer growth cones to their correct targets. Recent studies in *Drosophila* point to the importance of the combinatorial action of guidance molecules, and further show that selective fasciculation and defasciculation at specific choice points serve as a fundamental strategy for motor axon guidance. Here, I discuss how attractive and repulsive guidance cues cooperate to ensure the recognition of specific choice points that are inextricably linked to selective fasciculation and defasciculation, and correct pathfinding decision-making.

Keywords: axon guidance, *Drosophila*, guidance molecule, selective defasciculation, selective fasciculation

INTRODUCTION

Precise connections of neurons with their targets during neural development are responsible for the normal physiological and behavioral patterns of animals (Dorskind and Kolodkin, 2021; Engle, 2010). Neurons generated during embryonic and postnatal development extend axons that navigate along distinct paths to find their appropriate synaptic targets (Chédotal and Richards, 2010; Kolodkin and Tessier-Lavigne, 2011). Axon pathfinding is controlled by the coordinated action of attractive and repulsive cues (Tessier-Lavigne and Goodman, 1996). These opposing guidance cues act at either short-range or long-range (Kolodkin and Tessier-Lavigne, 2011; Tessier-Lavigne and Goodman, 1996). In general, long-range guidance cue molecules travel a long distance to bind to their cognate receptors expressed on growth cones, and then mediate either attractive or repulsive axon guidance (Kolodkin and Tessier-Lavigne, 2011; Tessier-Lavigne and Goodman, 1996). In contrast, short-range guidance cue molecules largely regulate axon–axon, axon–cell, and axon–extracellular matrix interactions (Kolodkin and Tessier-Lavigne, 2011; Tessier-Lavigne and Goodman, 1996). The question then arises of how these different types of axon guidance cues are integrated to steer growth cones toward their synaptic targets.

A wide range of axon guidance molecules, including four major classes of guidance cues-semaphorins, slits, netrins, and ephrins, have been discovered, and their guidance functions appear to be evolutionarily conserved across the animal kingdom (Bashaw and Klein, 2010; Dickson, 2002). Slits found in *Drosophila*, *Caenorhabditis elegans*, and vertebrates, are secreted guidance molecules that can serve as short-range repulsive cues (Brose et al., 1999; Hao et al., 2001; Kidd et al., 1999; Li et al., 1999). Netrins can mediate attractive axon guidance through their cognate receptors,

Received 18 May, 2021; revised 29 June, 2021; accepted 2 July, 2021; published online 13 August, 2021

eISSN: 0219-1032

such as Frazzled in *Drosophila*, UNC-40 in *C. elegans*, and DCC (Deleted in Colorectal Carcinomas) in vertebrates (Chan et al., 1996; Keino-Masu et al., 1996; Kolodziej et al., 1996). Semaphorins are a large family of transmembrane and secreted proteins that contain a conserved semaphorin domain (Pasterkamp, 2012). Most semaphorins can act as repulsive cues during neural circuit development (Kolodkin and Tessier-Lavigne, 2011). Vertebrate ephrins have been shown to mediate either repulsion or attraction in axon guidance, whereas *Drosophila* ephrin was reported to play an important role in axonal repulsion during neural development (Bossing and Brand, 2002; Kania and Klein, 2016; Liu et al., 2017). However, signaling mechanisms that function downstream of the guidance molecules and their crosstalk should diverge to meet the huge number of demands for wiring complex and diverse neural networks among animal taxa (Bashaw and Klein, 2010; Pasterkamp, 2012). To this end, understanding the molecular signaling mechanisms underlying axon pathfinding is one of the key goals of neural development. The *Drosophila* motor axon guidance is an excellent model system to study axon pathfinding mechanisms at the level of guidance receptors and their crosstalk, due to its simplicity and powerful genetic tools. This review summarizes the current understanding of molecular mechanisms underlying motor axon guidance in *Drosophila*, and discusses how the complex wiring observed in the nervous system of many higher organisms can be achieved by a limited number of guidance genes.

CELLULAR STRATEGY OF MOTOR AXON PATHFINDING IN THE *DROSOPHILA* NEUROMUSCULAR SYSTEM

Embryonic muscle development begins at stage 12, and somatic muscle patterning and specification are established before stage 16 (approximately 13 h after egg laying) (Bate, 1990). A repeating and identical pattern of 30 somatic muscle fibers is observed in each abdominal hemisegment A2-A7 (Fig. 1; Bate, 1990). In each hemisegment, these muscle fibers are innervated in a cell-type specific manner by 36 motor neurons that are generated and reside in the ventral nerve cord (VNC) (Fig. 1; Landgraf and Thor, 2006; Landgraf et al., 1997; Van Vactor et al., 1993). How can an individual axon, which extends from the cell bodies of motor neurons, follow the correct path to reach specific target muscle(s)? One of the most promising strategies is the control of motor axon pathfinding by the combined action of selective fasciculation at the initial step, and selective defasciculation at specific choice points (Fig. 2; Raper and Mason, 2010; Tessier-Lavigne and Goodman, 1996; Wang and Marquardt, 2013). In fact, two major nerve fascicles, namely the intersegmental nerve (ISN) and the segmental nerve (SN), and the transverse nerve (TN) as a minor nerve fascicle are observed within the VNC when late stage 16 embryos are immunostained with anti-Fas II antibody (Fig. 1; Grenningloh et al., 1991; Jeong, 2017; Van Vactor et al., 1993). This indicates that selective fasciculation of motor axons occurs before exiting the VNC. In the periphery, sequential defasciculation of the ISN at specific choice points creates three nerve branches called the ISN, ISNb, and ISNd, while selective defasciculation of the SN produces the SNa and SNc (Fig. 1; Landgraf and Thor, 2006; Van Vactor et al., 1993). Additional and sequential defasciculation of motor axons along each nerve pathway is also required to innervate particular target muscles (Fig. 2B; Jeong, 2017; Landgraf and Thor, 2006; Van Vactor et al., 1993).

SELECTIVE FASCICULATION AND DEFASCICULATION: THE FORMATION OF MOTOR NERVE BRANCHES IN THE PERIPHERAL NERVOUS SYSTEM

Selective fasciculation of motor axons during neural development is thought to require attractive interaction and specific adhesion among axons. Therefore, cell adhesion molecules (CAMs) that primarily mediate cell–cell adhesion function may contribute to the formation of motor nerve fascicles. One of the best candidate adhesion molecules for selective fasciculation is Fasciclin II (FasII), an immunoglobulin super-

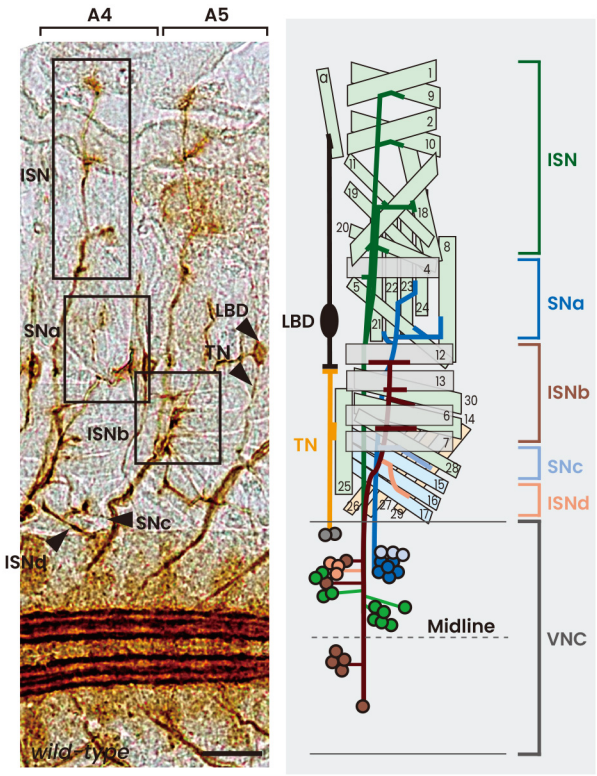

Fig. 1. Projection patterns of motor axons in the *Drosophila* embryos. In the left panel, late stage 16 wild-type embryos were stained with anti-FasII antibody, and then filleted to visualize motor axon projection patterns. Five major nerve branches, namely ISN, SNa, ISNb, SNc, and ISNd, and the transverse nerve (TN) as a minor nerve fascicle, are represented by the open boxes and arrowheads in abdominal segments, A4 and A5. An arrow indicates the lateral bipolar dendrite neuron (LBD) that makes synapses with the alary muscle (a). In the right panel, the schematic shows the cell bodies of embryonic motor neurons and their projection patterns in different colors, and their target muscles, which are numbered, in the VNC and peripheral nervous system. Scale bar = 15 μm.

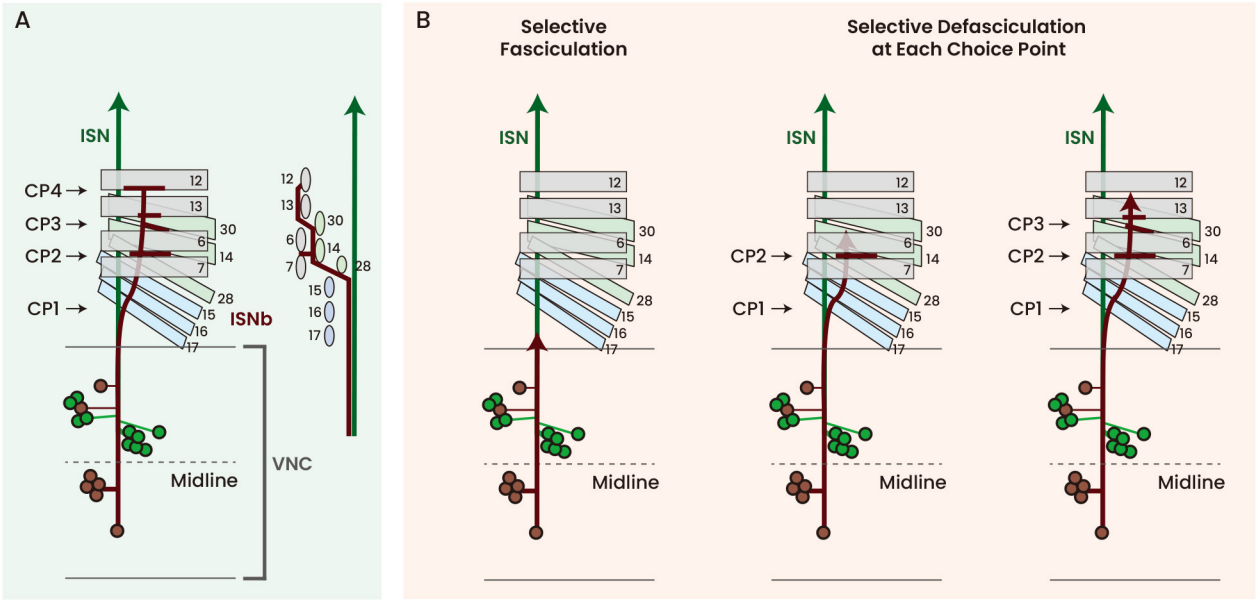

Fig. 2. Cellular strategy of motor axon guidance in the ISNb pathway. (A) Left schematic shows the ISNb motor axon projection patterns through the ventrolateral muscle fibers. Right schematic is a cross-sectional view of the stereotyped ISNb nerve pathway. Arrows indicate each defasciculation choice point (CP). (B) One of the most important mechanisms underlying motor axon pathfinding is selective fasciculation and defasciculation at specific choice points. At least seven ISNb motor neurons, which are born and lie at different positions in the VNC, extend their axons to form a fascicule with the ISN before exiting the VNC (left). After they recognize the first choice point (CP1), the ISNb axons defasciculate from the ISN. When the defasciculated ISNb nerve subsequently reaches the CP2, two axons selectively segregate from the main ISNb bundle (arrowhead in brown), and innervate both muscles 6 and 7 (middle). The main ISNb bundle grows dorsally to arrive at the CP3, which is located between muscles 6 and 13, thereby causing further selective defasciculation of three axons, and their innervation to muscles 13, 14, and 30 (right). Finally, at the CP4, the remaining two axons defasciculate, and extend in the opposite direction, to form synaptic connections with muscle 12 (shown in panel A).

family (IgSF) protein, since panneuronal overexpression of FasII resulted in a range of motor axon defasciculation defects due to hyperfasciculation (Figs. 3A and 3B; Lin and Goodman, 1994). In addition, loss-of-function studies showed that FasII is required to recognize a specific axon pathway through axon–axon attraction in the VNC, even though no motor axon pathfinding defects in the periphery are detected in *FasII* mutants (Grenningloh et al., 1991; Lin et al., 1994). However, the selective formation of five major nerve fascicles seen in the *Drosophila* peripheral nervous system (PNS) could not be explained merely by FasII-mediated cell adhesion function, because during neural development, FasII is expressed on all motor axons (Van Vactor et al., 1993). Another candidate adhesion molecule is Connectin (Con), a member of the leucine-rich repeat family, which is expressed on eight lateral and ventral muscle fibers, and also the motor axons that innervate these eight muscles (Nose et al., 1992). When Con is ectopically expressed on a subset of ventral muscles innervated by ISNb motor axons under the control of *Toll* enhancer element, the motor axon guidance phenotypes observed in the ISNb pathway are like those seen in FasII-overexpressing embryos (Fig. 3B; Lin and Goodman, 1994; Nose et al., 1994). As in the case of *FasII*, no significant axon pathfinding defects were found in *con* loss-of-function mutants (Nose et al., 1994), indicating functional redundancy in axonal fasciculation. Furthermore, some of the *Drosophila* CAMs, such as Neuroglian, Fasciclin III, and Capricious, which are expressed on either all or a subset of motor neurons, were also shown to be required for motor axon guidance (Abrell and Jäckle, 2001; Chiba et al., 1995; Hall and Bieber, 1997). These observations suggest that axon–axon adhesion is regulated by redundant and cooperative roles of multiple CAMs, and also demonstrate that CAMs are necessary, but do not seem to be sufficient for the selective fasciculation of motor axons during neural development.

One intriguing question remains as to what classes of guidance molecules collaborate with CAMs to control selective axon fasciculation. *In vivo* genetic analysis in *Drosophila* showed that transmembrane semaphorin-1a (Sema-1a), which is a member of the semaphorin protein family of axon guidance cues, has been implicated in the generation of three smaller nerve branches, including the ISN and ISNb via selective defasciculation (Yu et al., 1998). In *Sema-1a* mutant embryos, a small but significant fraction of ISNb motor axons fail to defasciculate from the ISN nerve, and thus bypass their normal target muscles, which are referred to as a "fusion bypass phenotype" (Fig. 3B; Yu et al., 1998). Similar fusion bypass phenotypes are also observed in *plexin A* (*PlexA*) mutant embryos (Fig. 3B; Winberg et al., 1998a). PlexA functions as a neuronal receptor for Sema-1a in motor axon pathfinding

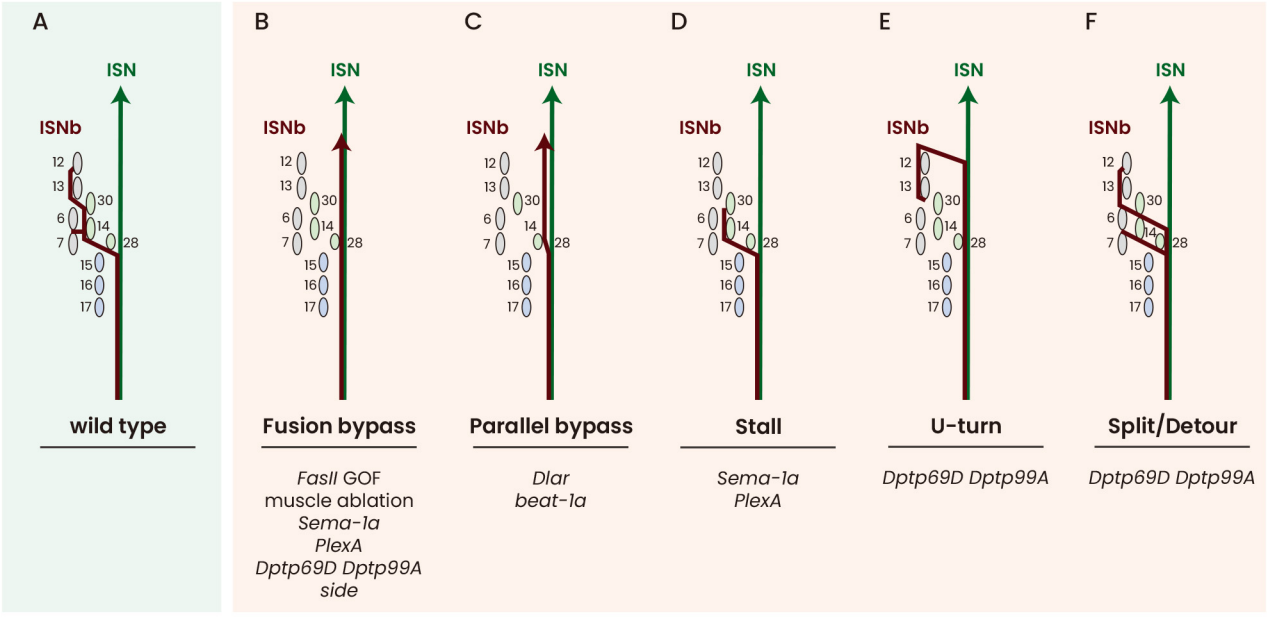

Fig. 3. ISNb motor axon guidance phenotypes in wild-type and mutant embryos. (A) In wild-type embryos, highly stereotyped projection patterns of ISNb motor axons are observed. Abdominal muscles are represented by numbers. (B) Fusion bypass phenotype is frequently found in embryos overexpressing FasII and genetically muscle-ablated embryos. This phenotype is also observed in many different types of mutant embryos, such as *Sema-1a*, *PlexA*, *Dptp69D Dptp99A*, and *side*. (C) Parallel bypass phenotype is often detected in embryos homozygous for either *Dlar* or *beat-1a*. (D) Stall phenotype is the most frequently observed defect in *Sema-1a* homozygous mutants. Similar but milder stall phenotype is found in embryos homozygous for *PlexA*. (E) U-turn phenotype is clearly observed in *Dptp69D Dptp99A* homozygotes. (F) In split/detour phenotype of *Dptp69D Dptp99A* mutants, some ISNb axons segregate from the ISN at an abnormal choice point and innervate muscles 13 and 12.

(Winberg et al., 1998a). In addition, it is well established that Sema-1a/PlexA-mediated signaling at specific choice points induces selective axon–axon repulsion (Jeong et al., 2012; 2017; Winberg et al., 1998a; Yu et al., 1998). Therefore, these results strongly suggest that Sema-1a/PlexA repulsive signaling contributes to the formation of the ISNb branch. This idea is further supported by strong genetic interactions between *Sema-1a* and *FasII*, and *conn* (Yu et al., 2000). The fusion bypass phenotype observed in *Sema-1a* mutants is enhanced in a synergistic manner by increased expression of FasII, but suppressed by loss-of-function mutations in *conn* and/or *FasII* (Yu et al., 2000). These findings support the idea that the selective formation of five nerve branches in the PNS depends on the combined action of Sema-1a-mediated repulsion and CAMs-induced adhesions.

Interestingly, highly penetrant fusion bypass phenotypes of the ISNb were also found in *Dptp69D Dptp99A* double mutant embryos (Fig. 3B; Desai et al., 1996). Both *Dptp69D* and *Dptp99A* encode receptor-linked protein tyrosine phosphatases (RPTPs) which during embryonic neural development are expressed on motor axons that include ISNb, ISN, and SNa axons (Desai et al., 1996; Hamilton et al., 1995). The motor axon guidance phenotypes observed in double mutant embryos suggest that these RPTP proteins play an important role in defasciculation at specific choice points (Desai et al., 1996). However, it is still unknown if Dptp69D and Dptp99A directly regulate axon–axon interactions, because to date, no ligands for these RPTPs have been discovered (Arzan Zarin and Labrador, 2019). The most valuable insights into the formation of five nerve branches that include ISN, ISNb, and SNa emerge from the genetic ablation of embryonic muscles (Landgraf et al., 1999; Prokop et al., 1996). In the absence of muscles, peripheral nerve branches, including the ISNb and SNa, fail to defasciculate from the main nerve bundles, resulting in a fusion bypass phenotype (Fig. 3B; Landgraf et al., 1999; Prokop et al., 1996). These genetic ablation studies further demonstrate that founder myoblasts produce specific defasciculation cues required for the formation of peripheral nerve branches (Landgraf et al., 1999). Taken together with the axon guidance functions of CAMs, Sema-1a/PlexA, and the two RPTPs mentioned above, these results suggest that the selective defasciculation of motor axons in the periphery is controlled by the cooperation between axon–axon adhesion and selective axon–axon repulsion in response to muscle-derived guidance cues at specific choice points.

SELECTIVE DEFASCICULATION AND PATHFINDING OF MOTOR AXONS FOR TARGETED MUSCLE INNERVATION

After five nerve branches that include ISN, ISNb, and SNa are formed in the periphery, each projects to its target muscle region, and then its bundled axons sequentially defasciculate at specific choice points to innervate target muscle fibers (Fig.

1). With respect to the selective defasciculation of motor axons for targeted muscle innervation, this review will for several reasons primarily focus on the ISNb motor axon guidance and its regulation. First, when stained with anti-FasII antibody, the visibility of the axonal projection pattern of the ISNb is greater than that of other nerve branches (Jeong, 2017; Van Vactor et al., 1993). In addition, the embryonic pattern of ventrolateral muscles 7, 6, 13, and 12, which are innervated by ISNb motor axons (Fig. 2A), is also well recognized under differential interference contrast (DIC) microscopy (Van Vactor et al., 1993). These patterns appear to be very helpful for identifying more subtle axon pathfinding defects. Second, no nonmuscle mesodermal cells, which could function as guidepost cells, have yet to be recovered in the peripheral ISNb pathway (Van Vactor et al., 1993). Therefore, axon–axon and axon–muscle interactions seem to play an important role in precise navigation of the ISNb motor axons. Third, it is likely that no pioneer axon is required for axon pathfinding and growth of the ISNb (Krueger et al., 1996; Van Vactor et al., 1993). This may suggest that each growth cone of the ISNb is able to differentially respond to guidance cues emanating from the surrounding, and also able to recognize its own target muscle(s) (Krueger et al., 1996). Fourth, loss-of-function studies have identified a relatively larger number of guidance molecules required for correct axon pathfinding in the ISNb pathway, compared to other nerve pathways (Arzan Zarin and Labrador, 2019). This could in part be ascribed to the absence of guidepost cell and pioneer axon in the ISNb (Krueger et al., 1996; Van Vactor et al., 1993). Finally, compared to other peripheral nerve branches, more severe and diverse guidance phenotypes seem to be observed in the ISNb (Desai et al., 1996; Fambrough and Goodman, 1996; Krueger et al., 1996; Sink et al., 2001; Van Vactor et al., 1993; Winberg et al., 1998a; Yu et al., 1998). Therefore, when one single gene or one copy of a gene is absent, subtle axon guidance errors could easily be detected. This is probably due to relatively low levels of genetic redundancy, which indicate that the individual guidance molecule retains distinct non-overlapping guidance functions.

The question then arises as to how motor axons can recognize defasciculation choice points. The recognition of specific choice points should be a prerequisite for the selective defasciculation of motor axons. Therefore, the fusion bypass phenotype observed in genetically muscle-ablated embryos indicates that muscle founder cells and muscle-derived cues seem to play an essential role in recognizing defasciculation choice points (Landgraf et al., 1999; Prokop et al., 1996). Moreover, the presence of a single founder cell in muscle-ablated embryos is sufficient to induce defasciculation of motor axons, further supporting this idea (Landgraf et al., 1999). The absence of both Dptp69D and Dptp99A results in highly penetrant fusion bypass phenotypes of the ISNb, which are like those of genetic muscle ablation (Fig. 3B; Desai et al., 1996; Landgraf et al., 1999). These findings may suggest that RPTP proteins expressed on motor axons both serve as signaling molecules required for the recognition of specific choice points, and seemingly the selective axon–axon defasciculation. In addition, Sema-1a/PlexA signaling may also be involved in the recognition of choice points due to the fusion bypass phenotypes, even though these phenotypes are occasionally observed in either *Sema-1a* or *PlexA* mutants (Fig. 3B; Winberg et al., 1998a; Yu et al., 1998). However, the most frequently observed guidance defect in *Sema-1a* mutants is a stall phenotype, in which the ISNb largely stalls between muscles 6 and 13 along the ISNb pathway (Fig. 3D; Jeong, 2017; Yu et al., 1998). This stall phenotype of *Sema-1a* mutants may reflect that the mutant ISNb axons still possess the ability to recognize specific choice points, and to respond differentially to multiple guidance cues emanating from muscles. Therefore, the differential responsiveness of ISNb axons to surrounding guidance cues, combined with the failure in axon–axon repulsion at specific choice points, could lead to stall phenotypes in *Sema-1a* mutants. Similar stall phenotypes were also detected in the absence of PlexA, suggesting that Sema-1a/PlexA signaling is required for the selective defasciculation of ISNb axons at specific choice points (Fig. 3D; Jeong et al., 2012; Winberg et al., 1998a; Yang et al., 2016). The secreted semaphorins Sema-2a and Sema-2b, which both function as ligands for PlexB, are required for proper defasciculation of the ISNb axons at the last choice point (Ayoob et al., 2006; Roh et al., 2016; Wu et al., 2011). Moreover, *Sema-2a* genetically interacts with *Netrin A*, *Netrin B*, and *FasII* in the control of target selection by the ISNb axons (Winberg et al., 1998b). These results demonstrate that Semaphorins/Plexins work together with other guidance molecules to regulate motor axon pathfinding and/or target recognition. On the other hand, numerous axon guidance molecules are also involved in the synaptic partner choice (Sanes and Yamagata, 2009; Sanes and Zipursky, 2020). In many cases, it is difficult to distinguish between their axon pathfinding and synaptic target recognition functions. Therefore, synaptic recognition within target muscle region will not be addressed here.

In addition to fusion bypass phenotypes, different types of pathfinding defects that include U-turn, split/detour, and split/stall phenotypes (Figs. 3E and 3F), were observed in *Dptp69D Dptp99A* double mutants, strongly suggesting that while Dptp69D and Dptp99A are also responsible for making the correct pathfinding decisions, they barely contribute to the recognition of muscle targets (Desai et al., 1996). In contrast, *Drosophila* Lar (Dlar), which is a member of type IIa RPTPs, plays an important role in recognizing muscle targets or fields (Johnson and Van Vactor, 2003; Krueger et al., 1996). In *Dlar* mutants, ISNb axons normally defasciculate from the ISN at the initial choice point, but frequently fail to enter their target muscle fields, and instead extend along the ISN pathway as a separate bundle, referred to as the "parallel bypass phenotype" (Fig. 3C; Krueger et al., 1996). Interestingly, this parallel bypass phenotype is robustly suppressed by the loss of *Dptp99A* (Desai et al., 1996). Given that no ISNb guidance defects were observed in *Dptp99A* null mutants (Hamilton et al., 1995), the negative genetic interactions between *Dlar* and *Dptp99A*, along with the positive genetic interactions between *Dptp69D* and *Dptp99A*, demonstrate that Dptp99A collaborates in a redundant manner with Dptp69D and Dlar to recognize defasciculation choice points and target muscles, and to make the correct pathway decisions. Other types of RPTPs, such as Dptp10D, Dptp52F, and Dptp4E, were shown to be involved in motor axon guidance

(Arzan Zarin and Labrador, 2019). *Dptp10D* interacts genetically with *Dptp69D*, *Dptp99A*, and *Dlar* in potentiating ISNb defaciculation (Sun et al., 2001). *Dptp52F* also acts together with *Dptp10D* and *Dptp69D* to regulate defasciculation of ISNb axons at specific choice points (Schindelholz et al., 2001). *Dptp4E Dptp10D Dptp69D* triple mutants show genetic enhancement of clump phenotypes observed in the ISNb pathway (Jeon et al., 2008). These complex genetic interactions among RPTPs reflect their functional redundancy, and further demonstrate that a combinatorial code of RPTPs controls the recognition of defasciculation choice points and the pathfinding decision-making.

Highly penetrant bypass phenotypes of the ISNb were also observed in either *beaten path-Ia* (*beat-Ia*) or *sidestep* (*side*) mutant embryos (Fambrough and Goodman, 1996; Sink et al., 2001). Both *beat-Ia* and *side* encode membrane-tethered immunoglobulin superfamily (IgSF) proteins that form a complex through direct protein–protein interaction (Li et al., 2017; Özkan et al., 2013; Siebert et al., 2009). Beat-Ia protein is mainly detected on motoneuron axons and growth cones, while Side is expressed on all muscles and sensory neurons as guidance substrates in the periphery (Fambrough and Goodman, 1996; Sink et al., 2001). Genetic and immunohistochemical analyses propose that Beat-Ia functions as a receptor to recognize Side-expressing muscles, and then to mediate growth cone turning of the ISN pioneer neurons (Siebert et al., 2009). However, it remains to be determined whether the same mechanism underlies the ISNb motor axon guidance, since *beat-Ia* and *side* mutations seem to result in a different bypass phenotype (Fambrough and Goodman, 1996; Sink et al., 2001). Parallel bypass phenotype often appears to be observed in *beat-Ia* mutant embryos, whereas fusion bypass phenotype is frequently found in *side* mutants (Figs. 3B and 3C; Fambrough and Goodman, 1996; Sink et al., 2001). In addition, *FasII* interacts genetically with *beat-1a*, but not with *side* (Fambrough and Goodman, 1996; Sink et al., 2001). Lastly, a negative genetic interaction between *beat-Ia* and *side* was observed in the larval innervation pattern of the ISNb neurons in muscles 12 and 13, but not in muscles 6 and 7 (Siebert et al., 2009). These observations might suggest that Beat-Ia and Side not only mediate a common signaling pathway, but also have distinct functions in the ISNb pathfinding. The fly genome contains a total 14 *beat*-like genes and 8 *side*-like genes, showing a Beat/Side protein interaction network (Li et al., 2017; Özkan et al., 2013; Pipes et al., 2001). Several other members of the Beat subfamily, including Beat-Ic, also appear to be involved in ISNb motor axon guidance (Pipes et al., 2001). In contrast, the functional roles of other Side subfamily members have not yet been characterized.

CONCLUSION AND PERSPECTIVES

One of the most prominent mechanisms underlying motor axon guidance is the selective fasciculation and defasciculation that occurs at specific choice points. At least two criteria for selective fasciculation should be fulfilled. First, the individual growth cones of motor neurons born at different locations must be guided to meet each other or pioneer axon(s). Second, these axons associate to form a fascicle via axon–axon attraction. Selective defasciculation of motor axons preferentially requires the recognition of defasciculation choice points in response to muscle-derived guidance cues. Multiple muscle-derived guidance cues should not only induce selective axon–axon repulsion, but also mediate attractive axon guidance in a differential manner. Once motor axons selectively defasciculate at specific choice points, additional guidance receptor molecules mainly expressed on the growth cones are involved in making a correct pathfinding decision for targeted muscle innervation in response to muscle-derived guidance cues.

One unsolved question in neural development is how the complex and stereotyped patterns of neural circuits observed in many higher organisms can be shaped by a limited number of guidance molecules (Dickson, 2002). This could be explained by several mechanisms that include combinatorial codes of guidance molecules, differential regulation of guidance receptors and their ligands, and the reiterative use of a limited set of guidance molecules (Bonanomi and Pfaff, 2010; Dickson, 2002; Pasterkamp, 2012). In addition, I here propose that selective fasciculation and defasciculation contribute much to the formation of complex and precise neural circuits with a relatively small number of guidance molecules, based on the following reasons. First, selective fasciculation of growing follower axons ensures their correct pathfinding between fasciculation and defasciculation points, even in the absence of the signaling mechanisms necessary for axon pathfinding. In the *Drosophila* ISNb nerve in which no pioneer axon is found, interestingly, the individual axon appears to respond to guidance cues present along the pathway. Therefore, fasciculated ISNb axons are enabled to respond better to surrounding guidance cues, so that they can arrive at defasciculation choice points in a more coherent manner. Second, the recognition of each defasciculation point, which seems to be mediated by combinatorial codes of guidance molecules, is tightly associated with selective defasciculation. These combinatorial mechanisms of guidance molecules might produce a much larger number of divergent cellular outputs for axonal segregation. Third, once axons selectively defasciculate at specific choice points, segregated axons enter their own target field to choose correct synaptic partners, largely through the short-range interactions between growth cones and potential targets (Sanes and Yamagata, 2009). These short-range interactions within a restricted target region are likely to require a lesser number of guidance molecules. Lastly, the myriad number of nerves and nerve tracts observed in the mammalian PNS and central nervous system (CNS) highlights the importance of selective fasciculation and defasciculation in their complex wiring (Chédotal and Richards, 2010; Luxey et al., 2020; Takahashi et al., 2010; Thiebaut de Schotten et al., 2015), and also suggests that the cellular strategy using selective fasciculation and defasciculation should increase the fidelity of axon pathfinding, even with the relatively small number of guidance molecules in mammals. Given that major classes of axon guidance molecules serve evolutionarily conserved guidance functions across the animal kingdom (Bashaw and Klein, 2010; Dickson, 2002; Kolodkin and Tessier-Lavigne, 2011), in summary,

the molecular and cellular mechanisms underlying selective fasciculation and defasciculation could be highly conserved from flies to mammals.

ACKNOWLEDGMENTS
This work was supported by a National Research Foundation of Korea (NRF) grant funded by the Korea government (MIST) (2018R1A2B6008037), and research funds of Jeonbuk National University in 2019.

AUTHOR CONTRIBUTIONS
S.J. contributed to the manuscript preparation.

CONFLICT OF INTEREST
The author has no potential conflicts of interest to disclose.

ORCID
Sangyun Jeong https://orcid.org/0000-0002-6203-0833

REFERENCES

Abrell, S. and Jäckle, H. (2001). Axon guidance of *Drosophila* SNb motoneurons depends on the cooperative action of muscular *Krüppel* and neuronal *capricious* activities. Mech. Dev. *109*, 3-12.

Arzan Zarin, A. and Labrador, J.P. (2019). Motor axon guidance in *Drosophila*. Semin. Cell Dev. Biol. *85*, 36-47.

Ayoob, J.C., Terman, J.R., and Kolodkin, A.L. (2006). Drosophila Plexin B is a Sema-2a receptor required for axon guidance. Development *133*, 2125-2135.

Bashaw, G.J. and Klein, R. (2010). Signaling from axon guidance receptors. Cold Spring Harb. Perspect. Biol. *2*, a001941.

Bate, M. (1990). The embryonic development of larval muscles in *Drosophila*. Development *110*, 791-804.

Bonanomi, D. and Pfaff, S.L. (2010). Motor axon pathfinding. Cold Spring Harb. Perspect. Biol. *2*, a001735.

Bossing, T. and Brand, A.H. (2002). Dephrin, a transmembrane ephrin with a unique structure, prevents interneuronal axons from exiting the Drosophila embryonic CNS. Development *129*, 4205-4218.

Brose, K., Bland, K.S., Wang, K.H., Arnott, D., Henzel, W., Goodman, C.S., Tessier-Lavigne, M., and Kidd, T. (1999). Slit proteins bind Robo receptors and have an evolutionarily conserved role in repulsive axon guidance. Cell *96*, 795-806.

Chan, S.S., Zheng, H., Su, M.W., Wilk, R., Killeen, M.T., Hedgecock, E.M., and Culotti, J.G. (1996). UNC-40, a *C. elegans* homolog of DCC (Deleted in Colorectal Cancer), is required in motile cells responding to UNC-6 netrin cues. Cell *87*, 187-195.

Chédotal, A. and Richards, L.J. (2010). Wiring the brain: the biology of neuronal guidance. Cold Spring Harb. Perspect. Biol. *2*, a001917.

Chiba, A., Snow, P., Keshishian, H., and Hotta, Y. (1995). Fasciclin III as a synaptic target recognition molecule in *Drosophila*. Nature *374*, 166-168.

Desai, C.J., Gindhart, J.G., Jr., Goldstein, L.S., and Zinn, K. (1996). Receptor tyrosine phosphatases are required for motor axon guidance in the *Drosophila* embryo. Cell *84*, 599-609.

Dickson, B.J. (2002). Molecular mechanisms of axon guidance. Science *298*, 1959-1964.

Dorskind, J.M. and Kolodkin, A.L. (2021). Revisiting and refining roles of neural guidance cues in circuit assembly. Curr. Opin. Neurobiol. *66*, 10-21.

Engle, E.C. (2010). Human genetic disorders of axon guidance. Cold Spring Harb. Perspect. Biol. *2*, a001784.

Fambrough, D. and Goodman, C.S. (1996). The *Drosophila* beaten path gene encodes a novel secreted protein that regulates defasciculation at motor axon choice points. Cell *87*, 1049-1058.

Grenningloh, G., Rehm, E.J., and Goodman, C.S. (1991). Genetic analysis of growth cone guidance in *Drosophila*: fasciclin II functions as a neuronal recognition molecule. Cell *67*, 45-57.

Hall, S.G. and Bieber, A.J. (1997). Mutations in the *Drosophila* neuroglian cell adhesion molecule affect motor neuron pathfinding and peripheral nervous system patterning. J. Neurobiol. *32*, 325-340.

Hamilton, B.A., Ho, A., and Zinn, K. (1995). Targeted mutagenesis and genetic analysis of a *Drosophila* receptor-linked protein tyrosine phosphatase gene. Rouxs Arch. Dev. Biol. *204*, 187-192.

Hao, J.C., Yu, T.W., Fujisawa, K., Culotti, J.G., Gengyo-Ando, K., Mitani, S., Moulder, G., Barstead, R., Tessier-Lavigne, M., and Bargmann, C.I. (2001). *C. elegans* slit acts in midline, dorsal-ventral, and anterior-posterior guidance via the SAX-3/Robo receptor. Neuron *32*, 25-38.

Jeon, M., Nguyen, H., Bahri, S., and Zinn, K. (2008). Redundancy and compensation in axon guidance: genetic analysis of the *Drosophila* Ptp10D/Ptp4E receptor tyrosine phosphatase subfamily. Neural Dev. *3*, 3.

Jeong, S. (2017). Visualization of the axonal projection pattern of embryonic motor neurons in *Drosophila*. J. Vis. Exp. *(124)*, 55830.

Jeong, S., Juhaszova, K., and Kolodkin, A.L. (2012). The control of semaphorin-1a-mediated reverse signaling by opposing pebble and RhoGAPp190 functions in *Drosophila*. Neuron *76*, 721-734.

Jeong, S., Yang, D., Hong, Y.G., Mitchell, S.P., Brown, M.P., and Kolodkin, A.L. (2017). Varicose and cheerio collaborate with pebble to mediate semaphorin-1a reverse signaling in *Drosophila*. Proc. Natl. Acad. Sci. U. S. A. *114*, E8254-E8263.

Johnson, K.G. and Van Vactor, D. (2003). Receptor protein tyrosine phosphatases in nervous system development. Physiol. Rev. *83*, 1-24.

Kania, A. and Klein, R. (2016). Mechanisms of ephrin-Eph signalling in development, physiology and disease. Nat. Rev. Mol. Cell Biol. *17*, 240-256.

Keino-Masu, K., Masu, M., Hinck, L., Leonardo, E.D., Chan, S.S., Culotti, J.G., and Tessier-Lavigne, M. (1996). *Deleted in Colorectal Cancer* (*DCC*) encodes a netrin receptor. Cell *87*, 175-185.

Kidd, T., Bland, K.S., and Goodman, C.S. (1999). Slit is the midline repellent for the Robo receptor in *Drosophila*. Cell *96*, 785-794.

Kolodkin, A.L. and Tessier-Lavigne, M. (2011). Mechanisms and molecules of neuronal wiring: a primer. Cold Spring Harb. Perspect. Biol. *3*, a001727.

Kolodziej, P.A., Timpe, L.C., Mitchell, K.J., Fried, S.R., Goodman, C.S., Jan, L.Y., and Jan, Y.N. (1996). *frazzled* encodes a *Drosophila* member of the DCC immunoglobulin subfamily and is required for CNS and motor axon guidance. Cell *87*, 197-204.

Krueger, N.X., Van Vactor, D., Wan, H.I., Gelbart, W.M., Goodman, C.S., and Saito, H. (1996). The transmembrane tyrosine phosphatase DLAR controls motor axon guidance in *Drosophila*. Cell *84*, 611-622.

Landgraf, M., Baylies, M., and Bate, M. (1999). Muscle founder cells regulate defasciculation and targeting of motor axons in the *Drosophila* embryo. Curr. Biol. *9*, 589-592.

Landgraf, M., Bossing, T., Technau, G.M., and Bate, M. (1997). The origin, location, and projections of the embryonic abdominal motorneurons of *Drosophila*. J. Neurosci. *17*, 9642-9655.

Landgraf, M. and Thor, S. (2006). Development of *Drosophila* motoneurons: specification and morphology. Semin. Cell Dev. Biol. *17*, 3-11.

Li, H., Watson, A., Olechwier, A., Anaya, M., Sorooshyari, S.K., Harnett, D.P., Lee, H.K., Vielmetter, J., Fares, M.A., Garcia, K.C., et al. (2017). Deconstruction of the beaten Path-Sidestep interaction network provides insights into neuromuscular system development. Elife *6*, e28111.

Li, H.S., Chen, J.H., Wu, W., Fagaly, T., Zhou, L., Yuan, W., Dupuis, S., Jiang,

Z.H., Nash, W., Gick, C., et al. (1999). Vertebrate slit, a secreted ligand for the transmembrane protein roundabout, is a repellent for olfactory bulb axons. Cell *96*, 807-818.

Lin, D.M., Fetter, R.D., Kopczynski, C., Grenningloh, G., and Goodman, C.S. (1994). Genetic analysis of Fasciclin II in *Drosophila*: defasciculation, refasciculation, and altered fasciculation. Neuron *13*, 1055-1069.

Lin, D.M. and Goodman, C.S. (1994). Ectopic and increased expression of Fasciclin II alters motoneuron growth cone guidance. Neuron *13*, 507-523.

Liu, L., Tian, Y., Zhang, X.Y., Zhang, X., Li, T., Xie, W., and Han, J. (2017). Neurexin restricts axonal branching in columns by promoting ephrin clustering. Dev. Cell *41*, 94-106.e4.

Luxey, M., Berki, B., Heusermann, W., Fischer, S., and Tschopp, P. (2020). Development of the chick wing and leg neuromuscular systems and their plasticity in response to changes in digit numbers. Dev. Biol. *458*, 133-140.

Nose, A., Mahajan, V.B., and Goodman, C.S. (1992). Connectin: a homophilic cell adhesion molecule expressed on a subset of muscles and the motoneurons that innervate them in *Drosophila*. Cell *70*, 553-567.

Nose, A., Takeichi, M., and Goodman, C.S. (1994). Ectopic expression of connectin reveals a repulsive function during growth cone guidance and synapse formation. Neuron *13*, 525-539.

Özkan, E., Carrillo, R.A., Eastman, C.L., Weiszmann, R., Waghray, D., Johnson, K.G., Zinn, K., Celniker, S.E., and Garcia, K.C. (2013). An extracellular interactome of immunoglobulin and LRR proteins reveals receptor-ligand networks. Cell *154*, 228-239.

Pasterkamp, R.J. (2012). Getting neural circuits into shape with semaphorins. Nat. Rev. Neurosci. *13*, 605-618.

Pipes, G.C., Lin, Q., Riley, S.E., and Goodman, C.S. (2001). The Beat generation: a multigene family encoding IgSF proteins related to the Beat axon guidance molecule in *Drosophila*. Development *128*, 4545-4552.

Prokop, A., Landgraf, M., Rushton, E., Broadie, K., and Bate, M. (1996). Presynaptic development at the *Drosophila* neuromuscular junction: assembly and localization of presynaptic active zones. Neuron *17*, 617-626.

Raper, J. and Mason, C. (2010). Cellular strategies of axonal pathfinding. Cold Spring Harb. Perspect. Biol. *2*, a001933.

Roh, S., Yang, D., and Jeong, S. (2016). Differential ligand regulation of PlexB signaling in motor neuron axon guidance in *Drosophila*. Int. J. Dev. Neurosci. *55*, 34-40.

Sanes, J.R. and Yamagata, M. (2009). Many paths to synaptic specificity. Annu. Rev. Cell Dev. Biol. *25*, 161-195.

Sanes, J.R. and Zipursky, L. (2020). Synaptic specificity, recognition molecules, and assembly of neural circuits. Cell *181*, 1434-1435.

Schindelholz, B., Knirr, M., Warrior, R., and Zinn, K. (2001). Regulation of CNS and motor axon guidance in *Drosophila* by the receptor tyrosine phosphatase DPTP52F. Development *128*, 4371-4382.

Siebert, M., Banovic, D., Goellner, B., and Aberle, H. (2009). *Drosophila* motor axons recognize and follow a Sidestep-labeled substrate pathway to reach their target fields. Genes Dev. *23*, 1052-1062.

Sink, H., Rehm, E.J., Richstone, L., Bulls, Y.M., and Goodman, C.S. (2001). *sidestep* encodes a target-derived attractant essential for motor axon guidance in *Drosophila*. Cell *105*, 57-67.

Sun, Q., Schindelholz, B., Knirr, M., Schmid, A., and Zinn, K. (2001). Complex genetic interactions among four receptor tyrosine phosphatases regulate axon guidance in *Drosophila*. Mol. Cell. Neurosci. *17*, 274-291.

Takahashi, E., Dai, G., Wang, R., Ohki, K., Rosen, G.D., Galaburda, A.M., Grant, P.E., and Wedeen, V.J. (2010). Development of cerebral fiber pathways in cats revealed by diffusion spectrum imaging. Neuroimage *49*, 1231-1240.

Tessier-Lavigne, M. and Goodman, C.S. (1996). The molecular biology of axon guidance. Science *274*, 1123-1133.

Thiebaut de Schotten, M., Dell'Acqua, F., Ratiu, P., Leslie, A., Howells, H., Cabanis, E., Iba-Zizen, M.T., Plaisant, O., Simmons, A., Dronkers, N.F., et al. (2015). From Phineas Gage and Monsieur Leborgne to H.M.: revisiting disconnection syndromes. Cereb. Cortex *25*, 4812-4827.

Van Vactor, D., Sink, H., Fambrough, D., Tsoo, R., and Goodman, C.S. (1993). Genes that control neuromuscular specificity in *Drosophila*. Cell *73*, 1137-1153.

Wang, L. and Marquardt, T. (2013). What axons tell each other: axon–axon signaling in nerve and circuit assembly. Curr. Opin. Neurobiol. *23*, 974-982.

Winberg, M.L., Mitchell, K.J., and Goodman, C.S. (1998b). Genetic analysis of the mechanisms controlling target selection: complementary and combinatorial functions of netrins, semaphorins, and IgCAMs. Cell *93*, 581-591.

Winberg, M.L., Noordermeer, J.N., Tamagnone, L., Comoglio, P.M., Spriggs, M.K., Tessier-Lavigne, M., and Goodman, C.S. (1998a). Plexin A is a neuronal semaphorin receptor that controls axon guidance. Cell *95*, 903-916.

Wu, Z., Sweeney, L.B., Ayoob, J.C., Chak, K., Andreone, B.J., Ohyama, T., Kerr, R., Luo, L., Zlatic, M., and Kolodkin, A.L. (2011). A combinatorial semaphorin code instructs the initial steps of sensory circuit assembly in the Drosophila CNS. Neuron *70*, 281-298.

Yang, D.S., Roh, S., and Jeong, S. (2016). The axon guidance function of Rap1 small GTPase is independent of PlexA RasGAP activity in *Drosophila*. Dev. Biol. *418*, 258-267.

Yu, H.H., Araj, H.H., Ralls, S.A., and Kolodkin, A.L. (1998). The transmembrane Semaphorin Sema I is required in Drosophila for embryonic motor and CNS axon guidance. Neuron *20*, 207-220.

Yu, H.H., Huang, A.S., and Kolodkin, A.L. (2000). Semaphorin-1a acts in concert with the cell adhesion molecules fasciclin II and connectin to regulate axon fasciculation in *Drosophila*. Genetics *156*, 723-731.