Motor Axon Pathfinding

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Motor neurons are functionally related, but represent a diverse collection of cells that show strict preferences for specific axon pathways during embryonic development. In this article, we describe the ligands and receptors that guide motor axons as they extend toward their peripheral muscle targets. Motor neurons share similar guidance molecules with many other neuronal types, thus one challenge in the field of axon guidance has been to understand how the vast complexity of brain connections can be established with a relatively small number of factors. In the context of motor guidance, we highlight some of the temporal and spatial mechanisms used to optimize the fidelity of pathfinding and increase the functional diversity of the signaling proteins.

Motor neurons residing in the brain stem and spinal cord extend axons into the periphery and are the final relay cells for locomotor commands. These cells are among the longest projection neurons in the body and their axons follow stereotypical pathways during embryogenesis to synapse with muscle and sympathetic/parasympathetic targets. Cellular studies of motor axon navigation in developing chick and zebrafish embryos have shown that motor neurons located at different rostrocaudal positions show specific preferences for axonal pathways (see Landmesser 2001; Lewis and Eisen 2003 for reviews). This early cellular research laid the foundation for molecular studies of motor axon guidance by establishing the concept that motor neurons are in fact a diverse cell population. The molecular studies covered in this article have sought to identify genetic

differences between motor neurons and to characterize the signaling pathways that underlie the specificity of motor axon targeting.

DEVELOPMENT OF MOTOR NEURON DIVERSITY: TRANSCRIPTION FACTORS

During embryonic development, a mapping relationship emerges that links the position of motor neuron cell bodies within the spinal cord to the location of their synaptic targets in the periphery. Motor neuron subtypes that extend axons together along major nerve pathways to limbs, for example, coalesce into longitudinally aligned columns in the spinal cord (Fig. 1A). Depending on the spinal cord level, each motor column is comprised of multiple motor pools, representing cells that innervate the same target, such as the pectoralis muscle

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Figure 1. Motor neuron subtype diversification. (A) Motor neurons are arranged into longitudinal columns along the rostrocaudal and dorsoventral axes of the spinal cord. Motor neurons that send axons together through proximal pathways share the same columnar organization. A continuous medial motor column (MMC) spanning all rostrocaudal levels contains motor neurons projecting to the axial muscles (dm, dermomyotome). Hypaxial motor column (HMC) neurons, which innervate body wall muscles (bw), and preganglionic motor column (PGC; termed column of Terni in chick) neurons, which innervate sympathetic ganglia (sg), occupy more lateral positions exclusively at thoracic levels. The lateral motor column (LMC) spanning brachial and lumbar spinal cord levels contains limb-innervating motor neurons and is further partitioned into lateral (LMCl) and medial (LMCm) divisions, which segregate neurons innervating muscles of the dorsal (dlb) and ventral (vlb) limb bud, respectively. A LIM-HD code imposes columnar motor neuron identities, whereas an intrasegmental HOX code ensures that neurons innervating the same muscle group segregate in discrete motor pools (B). Motor pools are variable in size, positioned at stereotypical locations, and are extensively overlapping along the rostrocaudal (R-C) and dorsoventral (D-V) axes of the neural tube. Cross-repressive interactions between rostral Hox6 activities and caudal Hox9 activities consolidate LMC and PGC columnar identities, respectively. Within the Hoxc6 domain, reciprocally exclusive activities of Hoxc5 and Hoxc8 determine the rostrocaudal position of motor pools innervating the scapulohumeralis anterior (Sca, purple), pectoralis (Pec, red), and flexor carpi ulnaris (FCU, blue) muscles. The intrasegmental segregation of FCU and Pec cells in the Hoxc6⁺ domain depends on the status of Hoxc6 in combination with other transcription factors (See text for details).

(Fig. 1B). The rostrocaudal, mediolateral, and dorsoventral positions of the cell bodies comprising each motor column and pool are conserved within a species and typically serve as the basis for naming spinal motor neuron subtypes (see Fig. 1 for details). At present, it remains unclear why motor neuron cell bodies assume such an organized pattern within the spinal cord, although it is assumed this cellular organization is somehow associated with the process of establishing proper inputs onto each motor neuron class to control complex locomotor behaviors.

Motor neuron identity is assigned through the stepwise activity of transcription factors whose expression is tuned to specific concentrations of inductive factors such as Sonic Hedgehog, Retinoic Acid, Fibroblast Growth Factors, and Wnt4/5 (Agalliu et al. 2009; for a review see Jessell 2000; Lee and Pfaff 2001). These signals trigger the expression of homeodomain (HD) transcription factors, leading to the formation of five genetically distinct progenitor cell domains marked by unique combinations of HD proteins. Within the spinal cord, all motor neurons appear to arise from the pMN domain marked by Pax6, Nkx6.1/6.2, and Olig2 (reviewed in Jessell 2000; Lee and Pfaff 2001). Newly generated motor neurons derived from the spinal cord pMN domain extend axons from the neural tube by taking a ventral-lateral trajectory, leading to the formation of ventral spinal roots. Interestingly, at hindbrain levels, motor neurons arise from two adjacent, but genetically distinct, progenitor domains—motor neurons extending axons out ventral roots such as cranial nerve XII are born from progenitors expressing Pax6, Nkx6.1/6.2, and Olig2 (like spinal motor neurons), whereas motor neurons exiting the neural tube from dorsal exit points arise from a more ventral progenitor cell domain that is labeled by Nkx2.2/2.9 and Nkx6.1/6.2 (Ericson et al. 1997). This distinction in dorsal-exiting and ventral-exiting motor neurons may be a carryover from the way these cells evolved, because dorsal exiting motor neurons are found in primitive chordates like *Amphioxus*, and ventral-exiting motor neurons seem to be a more recent addition to vertebrates

coinciding with the appearance of different types of muscles for locomotion (Fritsch and Northcutt 1993).

An abrupt switch in the complement of transcription factors occurs within cells as they transition from dividing progenitors to postmitotic motor neurons and initiate the process of axonogenesis. Two families of transcription factors have been implicated in the control of motor axon navigation: the 12 member LIM-HD class of transcription factors and the 39 member HOX family of HD proteins (Tsuchida et al. 1994; Dasen et al. 2005). Although direct target genes for these transcription factors are just beginning to be identified, genetic loss- and gain-of-function studies have established that these protein families function in a combinatorial manner to drive the diversification of motor neuron subtype identity, including regulating axonal growth. The specification of motor axon targeting by the LIM-HD and HOX transcription factors does not appear to be controlled by a static “code” that endows motor neurons with the ability to navigate the entire route to their targets; rather, the profile of transcription factors changes in many classes of motor neurons as they reach different axon choice points (Shirasaki and Pfaff 2002; Song and Pfaff 2005). Thus, two layers of gene regulation appear to control motor axon targeting—one for distinguishing individual motor neuron subtypes at the level of columns and pools (Fig. 1) and another layer of regulation that dynamically changes to coincide with the sequential choices that motor axons make as they encounter successive choice points.

One of the first examples of dynamic gene regulation during motor axonogenesis was linked to the way motor axons exit the neural tube. Motor neurons that extend axons ventrally transiently express a LIM-HD transcription factor code comprised of Isl1/2 and Lhx3/4, whereas neurons that exit dorsally lack Lhx3/4 during this initial phase of their axon growth (Sharma et al. 1998). Mice deficient in both Lhx3 and Lhx4 generate normal numbers of motor neurons, but ventral-exiting cells grow out of the neural tube from more dorsal sites.

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Despite the critical role of these redundant LIM-HD factors in axon targeting, the expression of *Lhx3/4* in most ventral-exiting motor neuron subtypes is remarkably short lived.

The reorganization of the combinatorial patterns of LIM-HD transcription factors coincides with the next phase in axon navigation whereby neurons within motor columns select major peripheral nerve pathways: MMC cells grow to axial targets, HMC to body wall musculature, PCC to sympathetic ganglia, and LMC to the limb (Fig. 1A). Genetic studies have provided strong evidence that the LIM-HD factors control motor column axon choices in the periphery of the embryo. First, the down-regulation of *Lhx3/4* seems to be critical for generating distinct motor columns. Only MMC neurons retain *Lhx3/4* expression once axons emerge from the neural tube, whereas the other classes (HMC, PGC, and LMC) extinguish the expression quickly (Fig. 1A). The down-regulation of *Lhx3* appears to be critical, because knockin mice engineered to retain *Lhx3* in HMC, PGC, and LMC cells result in a redirection of their axons toward the axial target of the *Lhx3*+ MMC cells (Sharma et al. 2000). In the lateral portion of the LMC, the down-regulation of *Lhx3* is accompanied by the extinction of *Isl1* and coactivation of *Isl2* and *Lim1* (*Lhx1*), which controls the proper growth of lateral-LMC axons into the dorsal limb (Kania et al. 2000).

Although direct binding of LIM-HD factors to the promoters of axon guidance receptors has yet to be established, *Lhx3* has been linked to the control of fibroblast growth factor (FGF) receptor 1 (*FgfR1*) in MMC neurons (Shirasaki et al. 2006), *Lim1* to *EphA4* in lateral-LMC cells (Kania and Jessell 2003), and *Isl1* to *EphB1* in medial-LMC neurons (Luria et al. 2008). The role of *FgfR1*, *EphA4*, and *EphB1* in motor axon guidance is described in more detail below. Taken together, these data indicate that LIM-HD transcription factors (1) function in a combinatorial manner, (2) act at several phases of motor neuron development, (3) control axon targeting from the neural tube, and (4) control motor column guidance decisions in the periphery of embryos.

An additional layer of complexity comes to light through the study of regulatory networks of HOX-class transcription factors, which also act as key determinants of motor neuron specification and pathfinding (Dasen et al. 2005). Discrete rostrocaudal domains of HOX factors have been found to coincide with motor columns as well as the finer divisions of columns that correspond to motor pools (Fig. 1B). *Hox6* paralogs are expressed by brachial LMC cells, *Hox9* paralogs by PGC cells, and *Hox10* paralogs by lumbar LMC neurons. Altering the pattern of HOX expression changes the rostrocaudal identity of motor neurons, leading to corresponding alterations in their nerve-muscle connectivity (Liu et al. 2001; Dasen et al. 2003; Shah et al. 2004; Dasen et al. 2005; Dasen et al. 2008; Wu et al. 2008). Because of cross-repressive interactions between rostral *Hox6* activities and caudal *Hox9* activities to establish the LMC and PGC columnar identities, the forced expression of *Hoxc9* in *Hoxc6*+ brachial motor neurons causes the cells to inappropriately innervate the PGC target—the sympathetic ganglia (Dasen et al. 2003). Although both LIM-HD and HOX genes have been linked to the control of motor column identity, it remains unclear just how these two families of transcription factors operate in the context of one another. In one known case, the promoter of the *Hb9* gene expressed by HMC, LMC, and MMC cells appears to be a direct target of LIM-HD and HOX factors (Lee et al. 2004; Nakano et al. 2005). Nevertheless, further work is needed to understand whether these transcription factors have epistatic relationships, synergize with one another, and/or interact to form higher-order regulatory complexes to regulate motor column axon pathfinding.

Functional studies of the HOX network have also linked these factors to the control of motor pool identity (Fig. 1B). As an example of the molecular logic governing motor pool identity, in the domain of *Hoxc6* activity within the brachial spinal cord, the reciprocally exclusive expression patterns of *Hoxc5* and *Hoxc8* segregate motor neurons into pools for the scapulohumeral anterior (Sca) muscle (*Hoxc5*+), flexor carpi ulnaris (FCU) muscle,

and the pectoralis (Pec) (*Hoxc8*+) muscle. Within the *Hoxc8* domain, the intrasegmental combinatorial activities of HOX-members, *Scip*, *Pea3*, and LIM-HD proteins serve as genetic markers that differentially label the FCU and Pec motor neurons (Dasen et al. 2005). The expression of *Nkx6.1* in hindlimb motor pools appears to be under the control of HOX genes (Dasen et al. 2005; Dasen et al. 2008). Mice deficient in *Nkx6.1* show defects in muscle nerve branch formation and target innervation, indicating that *Nkx6.1* is an intermediary step in the control of motor neuron-muscle connectivity (De Marco Garcia and Jessell 2008). In summary, the distal pathfinding choices that motor pools make to innervate individual muscles appear to be under the control of a complex combinatorial network of transcription factors from several different gene families. As with the LIM-HD factors, the HOX genes appear to function at several stages in motor neuron development to help establish motor column and motor pool identity.

MOTOR AXON GROWTH FROM THE NEURAL TUBE: THE INITIAL TRAJECTORY

A feature that distinguishes motor neurons from all other CNS neurons is their growth of axons into the periphery. Depending on the motor neuron class, the precise exit point selection can vary but generally is divided into two categories: those cells that grow ventrally (vMN) and those that exit dorsally (dMN). Motor neurons at spinal cord levels and somatic-classes of cranial motor neurons (i.e., those that innervate striated muscles) usually extend axons peripherally via ventral exit points, although there are exceptions such as the spinal accessory nerve and the trochlear nucleus, which both grow dorsally from the neural tube. Most dorsal-exiting motor neurons are found in the hind-brain and typically innervate targets that are derived from neural crest cells (classified as branchiomotor or visceromotor) (for a review, see Guthrie 2007). Embryonic dMN and vMN cells have different transcription factor profiles, suggesting they may use fundamentally different signaling pathways to extend axons from the neural tube (see previous).

An important step in understanding the guidance of motor axons from the neural tube came from the study of mice deficient in the G protein-coupled receptor *Cxcr4* and its cytokine ligand *Cxcl12* (Lieberman et al. 2005). *Cxcr4* is transiently expressed by vMN cells, whereas *Cxcl12* is detected in the mesenchymal cells flanking the spinal cord and caudal hind-brain (Fig. 2A). Disruption of *Cxcr4*:*Cxcl12* signaling causes many vMNs to project dorsally within the neural epithelium, and, at rostral spinal levels where dMN are located, to select dorsal exit points (Fig. 2B). It remains unclear whether *Cxcl12* is acting as a classic chemo-attractant or possibly modifying the activity of other signaling pathways (Chalasani et al. 2003; Chalasani et al. 2007).

Semaphorin-receptor mutants in the zebrafish *Plexin A3* gene (*sidetracked*) also display motor axon exiting defects (Palaisa and Granato 2007), providing evidence that multiple signals guide motor axons within the spinal cord. In fact, the midline is the source of many known guidance molecules implicated in motor axon growth, including Netrins, Semaphorins, Ephrins, and Slits (Colamarino and Tessier-Lavigne 1995; Guthrie and Pini 1995; Varela-Echavarría et al. 1997; Brose et al. 1999; Zou et al. 2000), which have specific effects on dMN and vMN axons. Thus, the precise set point of motor axon exiting may depend on which combinations of guidance cues each motor neuron subclass is programmed to respond. Nevertheless, the finding that in *Cxcr4* mutants some motor axons extend into the floor plate (Lieberman et al. 2005), which expresses multiple repellents, indicates that the midline cues alone are not sufficient to drive motor axons from the neural tube.

BOUNDARY CAP CELLS AND THE CONTROL OF NUCLEAR VERSUS AXON MIGRATION

The mechanisms that allow motor axons to pierce through the basement membrane around the neural tube are not well understood, but recent studies have begun to focus on the role of a unique subset of neural crest cells that ensure axons pass out of the neuroepithelium

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Figure 2. Motor axon outgrowth from the neural tube. (A) The initial trajectory of motor axon growth is defined by a combination of repulsive signals associated with the floor plate that orient axons away from the midline, and positive signals that attract motor axons toward their ventral exit points. Cxcl12 ligand (yellow) from the mesenchyme flanking the neural tube entices Cxcr4-expressing motor axons to cross the neuroepithelium. It is unclear whether Cxcl12:Cxcr4 signaling has chemoattractive properties per se or influences the activity of other axon guidance systems. Sema6A expressed by boundary cap cells clustered at ventral exit points activates Nrp-2 receptors to confine motor neuron cell bodies within the neural tube. Repulsive signals from the dorsal neural tube and dorsal root ganglia (DRG) may further shape the proximal motor axon trajectory. (B) In *Cxcl12* and *Cxcr4* mutant embryos, motor axons marked by expression of the Hb9::gfp transgene extend dorsally within the neuroepithelium (Reprinted, with permission, from Lieberam et al. 2005). Bottom right: schematic of the motor axon phenotypes of *Cxcl12* and *Cxcr4* knockout embryos. Note that the aberrant axonal trajectories observed in the mutants (purple) include invasion of the DRG.

while helping to prevent motor neuron cell bodies from leaving the CNS. These neural crest cells migrate to the ventral motor exit points and dorsal sensory entry points and are called boundary cap (BC) cells (Fig. 2A). Some of the genes that have been found to mark BC cells include *Krox20* (*Egr2*) (Wilkinson et al. 1989), *TrkB* (Ernfors et al. 1992), *monoamine oxidase B* (Vitalis et al. 2003), and *cadherin 7* (Niederlander and Lumsden 1996), although rostro-caudal and species-specific differences in these markers have been noted. The ablation of BC cells does not perturb motor axon outgrowth per se, but does result in motor nuclei becoming ectopically located in the periphery (Vermeren et al. 2003). The defect seems to arise when motor neuron cell bodies inappropriately translocate along their axons, resulting in the unusual settling of motor nuclei in peripheral spinal nerves. BC cells appear to be the source

of repulsive cues Sema6A and possibly Sema3s, and motor neurons express Neuropilin-2 (Npn-2) and Plexin-A1/A2 (Bron et al. 2007; Mauti et al. 2007) receptor complexes.

Sophisticated discrimination mechanisms must be in place to dissociate the behavior of axons from that of their cell bodies. For example, how do BC-derived semaphorins repel cell bodies but not axons? Apparently BC cells become associated with the ventral root after motor axons have extended beyond this point (Fraher et al. 2007). Convergent “desensitizing” interactions with other signaling systems might also contribute to the unresponsiveness of motor axons to BC-derived semaphorins, in spite of the expression of the appropriate receptors. In this context, it is noteworthy that Cxcr4:Cxcl12 signaling acting on motor axons has been shown to reduce the effect of repulsive molecules Sema3A and Slit-2. The attenuation

of repulsion by Cxcr4:Cxcl12 signaling appears to be mediated by inactivation of Rho, a necessary downstream effector of repulsive signaling by Semaphorins, Slits, and ephrin-As (Wahl et al. 2000; Chalasani et al. 2003; Chalasani et al. 2007). Furthermore, the modulatory activity of Cxcr4:Cxcl12 signaling on other guidance systems might explain why, on arrival in the periphery, the axons of Cxcr4- and Cxcl12-deficient vMNs frequently invade sensory ganglia, which appear to contain repulsive signals for spinal motor axons (e.g., ephrin-As) (Lieberam et al. 2005) (Fig. 2B).

SPINAL NERVES AND SEGMENTAL PATTERNING

The rostrocaudal segmentation of motor axons that give rise to the metameric organization of spinal nerves is imposed by the somites (Keynes and Stern 1984; Tosney 1988). After leaving the

neural tube, motor axons grow preferentially through the anterior-half of the somite (Fig. 3), which is also the preferred substrate for neural crest migration. In particular, the posterior and ventromedial sclerotome appear to express contact repellents that confine the movement of spinal motor axons and neural crest cells to the rostral-half of the sclerotome (Fig. 3A). Early biochemical purification procedures led to the discovery of peanut agglutinin (PNA)-binding glycoproteins of 48 and 55 kDa, localized in the posterior half-sclerotome, which account for most of the growth cone collapse-inducing activity of somite extracts (Stern et al. 1986; Davies et al. 1990; Oakley and Tosney 1991; Krull et al. 1995). Surprisingly, the identity of the PNA-binding chemorepellent factors has not been determined yet. Over the years, several other molecules have been proposed as candidate contact-repellents associated with the posterior half-sclerotome (for a review, see

Figure 3. Origin of the metameric pattern of spinal nerves. (A) After leaving the neural tube, motor axons converge into ventral roots, which show a characteristic repetitive rostrocaudal pattern of growth through the somites. The segmentation of motor nerves is imposed by repulsive signals localized in the posterior-half (P) of the sclerotome, thereby confining axons to the anterior somite (A) where attractive cues might be present. Neural crest cells, also sensitive to A-P somite signals, form sensory ganglia (DRG) adjacent to the anterior sclerotome. (R) rostral; (C) caudal; (FP) floor plate. (B) *Top*: Dorsal view of Hb9::gfp+ mouse embryo shows the metameric organization of ventral roots (asterisk) extending through the anterior-half of the somites. *Bottom*: Aberrant motor axon segmentation in the *Columbus* mouse mutant isolated in a forward genetic screening (Lewcock et al. 2007): motor axons fail to converge within the anterior sclerotome.

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Kuan et al. 2004). The recent analysis of single and compound mouse mutants of the semaphorin receptors Npn-1 and Npn-2 reveals that stepwise activation of Neuropilin signaling by *Sema3F* and *Sema3A* expressed in the caudal sclerotome is required for segmental patterning of motor and sensory components. *Sema3F*: Npn-2 signaling guides initial motor axon outgrowth through the anterior sclerotome, whereas later *Sema3A*:Npn-1 signaling is required for ventral root fasciculation (Roffers-Agarwal and Gammill 2009). These findings are further supported by the observation that Npn-1 signaling controls exit point selection and restricts motor nerve branching in zebrafish (Feldner et al. 2005).

Among other candidate repulsive systems, Ephrin-B1 in the caudal-sclerotome repels motor axons expressing EphB2 receptor in vitro. Nevertheless, mouse knockouts of *ephrin-B/EphB* show properly segmented spinal nerves, suggesting there are redundant signals (Orioli et al. 1996; Wang and Anderson 1997; Wang et al. 1998; Vermeren et al. 2000). The adhesion molecules F-spondin (Debby-Brafman et al. 1999; Tzarfati-Majar et al. 2001) and GPI-linked T-cadherin (Ranscht and Bronner-Fraser 1991; Fredette and Ranscht 1994; Fredette et al. 1996) are also expressed in the caudal sclerotome and have been shown to repel motor axons in vitro. Sclerotome-associated chondroitin sulphate proteoglycans (CSPGs), such as collagen IX and versican, might also play a role in segmental motor axon outgrowth (Landolt et al. 1995; Ring et al. 1995; Kubota et al. 1999; Perissinotto et al. 2000). Less is known about the role of axon attraction; however, hepatocyte growth factor (HGF) (Ebens et al. 1996) and Fgf (Shirasaki et al. 2006) are potential candidates to mediate the chemoattractive activity of the rostral sclerotomes (Hotary and Tosney 1996).

MMC PROJECTIONS TO AXIAL MUSCLES

Shortly after entering the periphery, motor axons encounter a proximal choice point where HMC and LMC neurons select a ventral pathway to innervate body wall and limb muscles, respectively, PCG neurons make a ventral turn

to reach the sympathetic ganglia, and MMC cells turn dorsally toward axial muscles (Fig. 4). Although surgical manipulation of chick embryos showed that the environment surrounding the axon trajectory rather than the final target is the main determinant of PGC path-finding (Yip 1987; Yip 1990), a target-derived chemoattractant was proposed to guide MMC projections based on the observation that after ablation of their target, MMC axons reorient toward the next nearest dermomyotome (Tosney 1987; Tosney 1988). This hypothesis has been supported by genetic experiments whereby reprogramming HMC, LMC, and PGC motor neurons to an MMC fate by ectopic expression of the LIM-HD transcription factor *Lhx3* was found to redirect their axons toward axial muscles (Sharma et al. 2000). In some cases, the reprogrammed cells took novel pathways toward the axial muscles as if they were responding to a chemoattractant (Sharma et al. 2000). This long-sought chemoattractive activity has been attributed, at least in part, to FGFs released by the dermomyotome and acting via the Fgfr1 receptor expressed by MMC cells (Shirasaki et al. 2006). Several lines of evidence suggest that FGFs are selective chemoattractants for MMC cells: (1) MMC cells are attracted toward sources of FGF4/8 when cultured in vitro, (2) ectopic-*Lhx3* expression was found to trigger the expression of Fgfr1 in the HMC, which correlated with the acquisition of HMC-attraction to FGF4/8, and (3) deletion of *Fgfr1* from mouse spinal neurons using Cre-lox genetics caused MMC axon guidance errors (Fig. 4C,D).

The penetrance of the *Fgfr1* mutant phenotype is incomplete (Shirasaki et al. 2006). This might be because of partial Cre-mediated recombination of the floxed-*Fgfr1* allele, or could reflect the contribution of additional guidance cues for directing MMC cell axons to axial muscles. Recent studies of ephrin-A:EphA signaling suggest that MMC cells are repelled from inappropriate areas, such as the limb and DRG (Gallarda et al. 2008). MMC motor neurons express EphA3 and EphA4 receptors and are strongly repelled by ephrin-As in vitro. Double knockouts of *EphA3* and *EphA4* result in MMC misprojections into the DRG,

Figure 4. Motor axon navigation toward the axial muscles. (A) In the immediate periphery of the neural tube, the axons of MMC neurons expressing EphA3/A4 receptors extend through a permissive channel demarcated by repulsive ephrin-A ligands associated with the DRGs and ventral mesenchyme. FGF4/8 secreted by the dermomyotome collaborates to attract Fgfr1-positive MMC axons. Axons of other motor neuron subtypes do not respond to these signals and continue to extend ventrally. (B) In *EphA3/EphA4* double mutants, GFP-labeled motor axons (Tg Hb9::gfp) fail to respond to repulsive ephrin-As in the DRG and invade the sensory ganglia (white arrow), marked by Isl1 expression (red) (Reprinted, with permission, from Gallarda et al. 2008). (C) Likewise, neuron-specific ablation of Fgfr1 leads to motor axon misprojection into the DRG (white arrow) (Reprinted, with permission, from Shirasaki et al. 2006). (D) Schematic representation of the phenotypes observed in *EphA3/A4* and *Fgfr1* mutants: MMC motor axons fail to innervate the axial muscle and aberrantly grow into the DRG (purple).

reminiscent of the phenotype observed when *Fgfr1* is mutated (Fig. 4B–D) (Shirasaki et al. 2006; Gallarda et al. 2008). Taken together, these findings indicate that target-derived FGFs attract MMC axons through a permissive channel between the ephrin-A repulsive environments of the DRG and flank tissue. Although Fgfr1- and EphA-signaling may operate independently on the actin cytoskeleton to trigger attraction and repulsion, respectively, it is also possible that they function cooperatively. Recent experiments in cell lines have unmasked physical interactions between Fgfr1 and EphA4 and these receptors are found to potentiate each other's

signaling (Yokote et al. 2005). Although further studies on MMC motor neurons are needed to resolve whether Fgfr1 and EphA4 function as part of a hetero-signaling complex as observed in glioma cells, the two receptors appear to share downstream effectors such as ephexin1, a guanine nucleotide exchange factor (GEF) for Rho-mediated axon repulsion (Shamah et al. 2001; Sahin et al. 2005; Zhang et al. 2007). Repulsive Sema3A ligands released from the DRGs might also influence the trajectory of MMC axons, helping to keep them away from the ganglia. Paradoxically, the target of MMC cells, the dermomyotome, also expresses Sema3A. Interestingly,

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the sensitivity of motor axons to *Sema3A* seems to be set by an autocrine pathway. MMC expression of *Sema3A* limits the surface availability of Npn-1 on the growth cones of these cells, thereby desensitizing them to the *Sema3A* produced by the surrounding tissues (Moret et al. 2007). Precise temporal regulation of this system might help to modulate motor neuron responses to semaphorins at each choice point.

LMC GUIDANCE WITHIN THE LIMB

The axons of LMC neurons ignore the proximal choice point of MMC axons and grow in tight fascicles to the base of the limb. Here, axons from multiple spinal nerves converge: at forelimb levels, segments C1–C5 join to form a cervical plexus and C5–T1 form the brachial plexus. At hindlimb levels, L1–L4 join to form the lumbar plexus and L4–S4 comprise the sacral plexus. Within each plexus, axons pause during development before emerging along specific nerve tracts into either the dorsal or ventral portions of the limb bud. Thus, in the plexus regions, LMC axons make key navigational choices and undergo a significant redistribution involving cell–cell contacts mediated by cell adhesion molecules L1 and NCAM. Interfering with these adhesion systems by reducing the levels of polysialic acid-modified NCAM is found to enhance motor axon fasciculation and causes projection errors (Landmesser et al. 1990; Tang et al. 1992; Tang et al. 1994).

As LMC axons emerge from the plexus region, they select either a dorsal trajectory, where most extensor muscles develop, or a ventral path toward developing flexors. This dorso-ventral choice is made with great precision—motor neurons that settle in the lateral division of the LMC (LMCl) innervate the dorsal limb, whereas medial LMC (LMCm) motor neurons grow only to the ventral limb (Landmesser 1978; Lance-Jones and Landmesser 1981a; Tosney and Landmesser 1985a; Tosney and Landmesser 1985b). Surgical rotations of the limb before innervation, or targeted gene deletions that alter dorsoventral limb patterning lead to compensatory changes in LMCl and LMCm pathfinding. Taken together, these

findings suggest that cues at the base of the limb guide LMC axons (Lance-Jones and Landmesser 1981b; Ferguson 1983; Whitelaw and Hollyday 1983; Tosney and Landmesser 1984; Kania and Jessell 2003; Luria and Laufer 2007; Luria et al. 2008).

The restricted distribution of ephrin-A ligands in the ventral limb mesenchyme and EphA4 receptors in dorsally projecting LMCl axons provided a first clue for the involvement of the ephrin-A:EphA system in limb innervation (Fig. 5A) (Ohta et al. 1996; Eberhart et al. 2000). Misexpression experiments in chick (Eberhart et al. 2002) and knockout approaches in mouse embryos (Helmbacher et al. 2000; Kania and Jessell 2003) indicate that EphA4 mediates repulsion of LMCl axons from the ventral domain of ephrin-A expression through a Src family kinase-dependent pathway (Kao et al. 2009). In *EphA4* mutant embryos, LMCl axons project aberrantly into the ventral half of the hindlimb, causing thinning of the peroneal nerve, the main dorsal nerve of the hindlimb (Fig. 5B,C). The expression of EphA4 in LMCl neurons requires the LIM-HD transcription factor *Lim1* (Kania and Jessell 2003). Interestingly, both *EphA4* and *Lim1* mutants display limb innervation defects, but the phenotypes differ slightly. In *EphA4* mutants, LMCl neurons grow incorrectly into the ventral limb, whereas *Lim1* mutants have randomized growth into the dorsal and ventral limb.

Because *Lim1* is a transcription factor, it would not be surprising if *Lim1* mutants had changes that included other guidance molecules in addition to EphA4. However, it is also possible that EphA4 has both motor neuron autonomous and nonautonomous functions, whereas *Lim1* function is restricted to LMCl neurons. Namely, EphAs are also expressed in the dorsal limb mesenchyme where they could serve as “ligands” for ephrin-As expressed by LMCl axons (i.e., “reverse signaling”) (Fig. 5A). Unlike the repulsive effect of EphA-mediated forward signaling, activation of ephrin-A reverse signaling promotes motor axon growth (Marquardt et al. 2005). In principle, these bidirectional interactions could occur simultaneously in individual growth cones because the coexpressed

Figure 5. Motor axon navigation into the limb. (A) *Left:* Isl1-positive LMCm axons (purple) expressing Npn-2 and EphB1 are repelled by Sema3F and EphrinB in the dorsal limb and select a ventral trajectory. *Right:* A combination of repulsive and attractive signals orient Lim1-positive LMCI axons (green) to the dorsal limb muscles. Ephrin-As in the ventral mesenchyme trigger “forward” repulsive signaling through axonal EphA4, whereas EphAs localized in the dorsal limb may elicit attractive “reverse” signaling through axonal ephrin-As. GDNF localized at the base of the limb controls the dorsal trajectory of LMCI axons expressing c-Ret. (B) *Left:* Disruption of Sema3F:Npn-2 or ephrin-B:EphB1 signaling leads to dorsal projection of ventrally fated LMCm axons. *Right:* Ablation of EphA4 or interference with GDNF:c-Ret signaling causes dorsally projecting LMCI axons to select a ventral trajectory. (C) Dorsal view of hindlimb innervating motor axons visualized with the Hb9::gfp transgene in e12.5 mouse embryos. The dorsal (peroneal) nerve becomes thinner in *EphA4* mutants because of the misprojection of LMCI axons into the ventral limb (the ventral nerve is out of the plane of focus).

EphA and ephrin-A proteins localize to distinct protein islands within the neuronal membrane so that they are available to interact with ligands encountered on different cells (Marquardt et al.

2005). Thus, *EphA4* null mutations might affect both forward and reverse signaling in LMCI neurons, whereas *Lim1* mutants might preserve the attraction LMCI axons have for the dorsal

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limb from EphA-ephrin-A reverse signaling. These observations raise the intriguing possibility that EphA forward and ephrin-A reverse signaling function coordinately to determine the dorsal choice of LMCI axons. In support of this bidirectional signaling model for LMCI guidance, down-regulation of EphA7 in the dorsal mesenchyme of the chick wing leads to pathfinding errors of dorsally projecting motor axons (Araujo et al. 1998).

The role of ephrin-A:EphA signaling in LMCI neurons is paralleled by the activity of ephrin-B:EphB signaling in LMCm neurons guided to the ventral limb. LMCm cells express EphB receptors, which mediate repulsion from ephrin-B2 ligands in the dorsal limb (Fig. 5A, B) (Luria et al. 2008). Expression of EphB1, the main EphB isoform in LMCm neurons, requires the LIM-HD protein Isl1, expressed by LMCm but not LMCI neurons. In mouse embryos with limb-specific deletion of *ephrin-B2* or compound mutations in *EphB1/2/3* genes, LMCm axons select an aberrant dorsal trajectory, whereas the dorsal projection of LMCI axons is unaffected.

Other guidance systems act in parallel or perhaps synergistically with ephrin:Eph signaling to increase the fidelity of dorsoventral navigation at the base of the limb. Genetic approaches in mouse and chick demonstrate that repulsive *Sema3:Neuropilin* signaling contributes to LMC axon navigation (Huber et al. 2005). *Npn-2* receptor expressed by LMCm neurons mediates repulsive signaling activated by *Sema3F* in the dorsal limb to divert their axons ventrally (Fig. 5A, B). In contrast, the high affinity *Sema3A* receptor *Npn-1* is expressed throughout the entire brachial LMC, and disruption of *Sema3A-Npn-1* signaling impairs several aspects of limb innervation, including the timing of in-growth, axon fasciculation, and bifurcation of motor projections of both divisions of the LMC.

In addition, limb-derived growth factors such as glial cell line-derived neurotrophic factor (GDNF) and HGF play a role in motor axon navigation. GDNF is localized at the base of the limb when LMC axons are selecting their dorsoventral trajectory into the limb bud. LMCI

axons express the GDNF receptor c-Ret, which is required for their growth into the dorsal limb (Fig. 5A, B) (Kramer et al. 2006). In mouse mutants of *GDNF* and *c-Ret* LMCI axons are misrouted to the ventral limb, similar to the phenotype displayed by *EphA4* mutants (Fig. 5B). It remains to be determined whether the GDNF:c-Ret pathway provides an attractive signal for LMCI axons or modulates the activity of other guidance pathways involved in limb innervation. Likewise, HGF is produced by the limb mesenchyme and LMC axons express the HGF receptor c-Met as they extend toward the limb and branch to reach muscle targets (Ebens et al. 1996; Yamamoto et al. 1997). In spite of the potent chemoattractive activity of HGF in vitro, the in vivo functions of this signaling pathway are less well characterized but may contribute to muscle-specific nerve targeting (Ebens et al. 1996; Caton et al. 2000).

BRANCHING OF MOTOR POOLS ON INDIVIDUAL MUSCLES

As a result of communal responsiveness to guidance molecules imposed by motor pool-specific transcriptional programs, the axons of neurons clustered in the same pool within the spinal cord project to the same muscle with high accuracy (Landmesser 2001). Once at the muscle, individual growth cones appear to respond to specific environmental cues that ensure axons grow to the appropriate muscle subregion (Tosney and Landmesser 1985c). The observation that the rostrocaudal position of motor pools is systematically mapped onto the rostrocaudal axis of the muscles suggests that matching phenomena between complementary positional labels on motor axons and their targets ensure extensive synaptic selectivity (Laskowski and Sanes 1987; Wang et al. 1999 and references within). Remarkably, the positional information is retained in reinnervation and muscle transplantation experiments where the contribution of classic guidance phenomena can be excluded (for references, see Sanes and Lichtman 1999).

The identity of nerve–muscle recognition molecules is still largely unknown. However,

the impaired neuromuscular topography of mouse mutants either lacking ephrin-A2/A5 or overexpressing ephrinA5 in skeletal muscles implicates the ephrin-A/EphA system in the process of nerve-muscle matching (Feng et al. 2000; Lampa et al. 2004). Work in *Drosophila* has begun to identify the players involved in the matching mechanisms (reviewed in Sanes 2009 and Ruiz-Canada and Budnik 2006). Leucine-rich repeat proteins, cell adhesion molecules, members of the immunoglobulin and cadherin superfamilies, as well as classic attractive and repulsive molecules such as Netrin/DCC and semaphorins are promising candidates to be tested in vertebrates for a role in the formation of highly stereotyped nerve-muscle projection patterns.

An example of the logic that links motor pool-specific branching onto muscles and the transcriptional profiles that define pool identity is offered by the ETS-class transcription factor Pea3 (Lin et al. 1998; Arber et al. 2000). Pea3 is activated by a retrograde mechanism when motor axons encounter GDNF around the cutaneus maximus (CM) and latissimus dorsi (LD) muscles. The induction of Pea3 by GDNF activates a motor neuron branching program, thus mice lacking GDNF display defects in CM and LD muscle innervation (Lin et al. 1998; Haase et al. 2002; Livet et al. 2002; Price et al. 2002). Interestingly, knockin mice of the GDNF receptor c-Ret harboring a point mutation in the multidocking site tyrosine-1062 have defective muscle arborization in the hindlimb, but relatively normal LMCI neuron pathfinding into the dorsal limb compared with *c-Ret* null mutants (see LMCI pathfinding earlier) (Gould et al. 2008). The dissociation of axon branching from axon guidance suggests that c-Ret mediates several distinct aspects of motor neuron connectivity using separate effector pathways.

At the heart of controlling growth cone turning and axon branching are signaling events that regulate cytoskeletal organization at a subcellular level. Recent studies of motor axon targeting identified the evolutionarily conserved microtubule-associated protein Phr1 in a mouse ENU screen for motor pathfinding errors (Schaefer et al. 2000; Wan et al. 2000;

DiAntonio et al. 2001; Nakata et al. 2005; Collins et al. 2006; Lewcock et al. 2007). In the absence of Phr1, motor axons grew normally and were able to respond to guidance cues in vitro. Nevertheless, embryos lacking Phr1 display numerous pathfinding errors. The basis for the axon guidance phenotypes of *Phr1* mutants requires further investigation, but seems to be linked to microtubule destabilization and the defective morphology of growth cones. Phr1 controls microtubule stability in the axon shaft by preventing the accumulation of MAP kinase activities through the degradation of DLK (dual leucine zipper kinase) (Lewcock et al., 2007). Because Phr1 has been implicated in neuromuscular junction (synapse) formation and axon branching, this protein may help to coordinate axon guidance, axon branching, and synaptogenesis through a common cytoskeletal signaling pathway (Bloom et al. 2007; Lewcock et al. 2007). In addition, the recent discovery that in *C.elegans* the DLK-1 pathway is required for axon re-growth after lesion suggests a role for Phr1 signaling in axon regeneration (Hammarlund et al. 2009; Yan et al. 2009).

CONCLUDING REMARKS

The developmental program outlined above proceeds through the stepwise acquisition of restricted transcriptional identities, which translates into progressively more specific repertoires of guidance molecules that control the stereotypical projection of each motor pool. Although this rather deterministic view may explain with good approximation the selection of the major motor axon paths, it may not entirely account for the extreme diversification and specificity observed in motor pool-muscle matching. Indeed, activity-dependent mechanisms and axon “crowding” interactions also appear to have an impact on motor axon targeting (Sharma et al. 2000; Hanson and Landmesser 2004). Particularly significant in this regard is the contribution of transaxonal signaling as a general strategy to, (1) organize pioneer-follower hierarchies between co-extending axons (Pittman et al.

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2008), (2) presort axons that establish topographic connections onto the same target (Imai et al. 2009), and (3) segregate heterotypic axons (Gallarda et al. 2008). In the motor system, axon–axon communication might explain the incomplete penetrance of the axon rerouting induced by *Lhx3* overexpression, where all motor neurons are converted to the axial subclass (Sharma et al. 2000).

An emerging problem in developmental neurobiology is to explain how complex neuronal circuits can be assembled with the limited number of known guidance molecules (Dickson 2002). This is apparent in the study of motor system development, where a high degree of pathfinding diversity is generated through the repetitive use of a restricted repertoire of guidance molecules by motor neuron subtypes that project to different muscle targets. In some cases, this context-dependent use of the same signaling module seems to require sophisticated spatial and temporal regulation. For example, ephrin-A ligands in tissues flanking the neural tube divert EphA3/4-expressing MMC axons dorsally toward axial muscles, whereas LMC axons grow through this ephrin-A⁺ environment toward the limb (Fig. 4). On arrival at the limb, LMCI neurons are repelled from the ephrin-A⁺ ventral mesenchyme (Fig. 5). Thus, the spatial/temporal regulation of ephrin-A responsiveness is a key aspect of LMCI pathfinding. Although the mechanistic basis for this temporal switch remains unknown, there are many possible ways EphA4 signaling might be controlled in LMCI axons. For example, *EphA4* transcription may be delayed in LMC neurons relative to MMC cells. This delay might be cell-intrinsic or could be initiated by retrograde signals encountered by LMCI axons at the base of the limb. Alternatively, temporal regulation of EphA4 translation, insertion into the membrane, or downstream effectors like ephexin (Sahin et al. 2005) might account for the ability of LMC neurons to first ignore ephrin-A ligands proximally but acquire responsiveness distally. The well-established waiting period of LMC axons within the plexus regions at the base of the limbs might provide time for the reprogramming of LMC responsiveness to guidance cues

(Lance-Jones and Landmesser 1981a; Tosney and Landmesser 1985b; Wang and Scott 2000; Huber et al. 2005).

An additional mechanism for generating axon pathfinding diversity might derive from the use of separate signaling modules, orchestrated by distinct effectors downstream of the guidance receptors. Future studies of the pathways linking signaling at the growth cone membrane to cytoskeletal reorganization will be helpful in elucidating additional regulatory points for controlling the spatial and temporal modulation of axon growth. However, after identifying new checkpoints, it may still prove difficult to rationalize how a limited repertoire of guidance molecules is used to wire the brain. A better understanding of how growth cones integrate information from multiple guidance cues will undoubtedly be helpful in this regard.

In addition to motor axon pathfinding at the periphery, the classic guidance molecules discussed above are beginning to be implicated in the establishment of patterns of connectivity within the spinal cord. Gating of *Sema6A*:*PlexinA4* signaling by FARP1, a Rho-GEF selectively expressed by LMC neurons, activates a subtype-specific program of dendrite arborization (Zhuang et al. 2009), which is essential for the proper assembly of sensory-motor circuits (Vrieseling and Arber 2006). Synaptic specificity is further ensured by repulsive *Sema3A*:*PlexinD1* signaling between sensory afferents and motor neuron pools, which prevents the formation of inappropriate connections (Pecho-Vrieseling et al. 2009). Because of the well-defined neuroanatomy of motor neurons, extensive characterization of genetic markers for individual subclasses, and the emerging identification of the key receptors and ligands, studies of motor neurons will continue to serve as a useful system for understanding general principles for establishing the intricate pattern of connections within the brain.

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