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Guidance of Developing Motor Neurons into the Periphery

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by

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Abstract

Motor neurons (MNs) are a diverse class of cells that originate in the central nervous system and project to muscles in the periphery. The development of all MNs subtypes begins with Sonic Hedgehog (Shh) signaling in the spinal cord and then progresses to increasingly specific guidance signaling systems. MN subtypes are classified by the combined activities of transcription factors and molecular signaling systems that determine axon trajectory. Molecular cues have been attributed to the organization of MNs into columns within the spinal cord and then into specific motor pools. These cues have also been shown to be responsible for axon guidance into the periphery and important for axons to innervate the correct targets. Despite advances in research on specific guidance cues used by different subdivisions of MNs, it is still not fully understood how signaling systems work with one another in the same environment to guide different classes of MNs to their respective muscle targets.

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Introduction

The nervous system is composed of many different populations of neurons, each of which projects axons along unique pathways to locate their respective targets. Developing axons project toward their destinations as guided by growth cone dynamics in response to various signaling systems. Motor neurons (MNs) are one of the only neuronal populations originating in the central nervous system (CNS) to extend their axons into a peripheral environment (Lieberam et al., 2005). MN axons are also some of the longest projection neurons as their trajectories arise in the spinal cord and end at various muscle targets (Bonanomi and Pfaff, 2010).The development of MNs has been extensively studied and many signaling systems and transcriptional profiles have been linked to MN subtype and target selection.

MNs develop in a stepwise fashion, with major choice points being controlled by different combinations of signaling systems. Most of the studies on MN guidance have focused on transcription factors and signaling systems that set up early organization and define MN subtype identities. Less research has been compiled on the molecular signaling systems that guide the development of MN subsets towards their targets in the periphery. The aim of this paper is to examine the numerous molecular guidance systems that operate during MN pathfinding.

Establishment of Generic Motor Neuron and Class Identity

Early MN generic and class identity is controlled by two main patterning systems, one that acts along the dorsoventral axis and one that operates along the rostrocaustal axis of the neural tube. Generic MN fate specification is initially induced by a dorsoventral

gradient of Sonic Hedgehog (Shh) signaling in the ventral neural tube (Agalliu et al., 2009). The other major signaling system consists of the graded activities of fibroblast growth factor (FGF) and retinoids which induce Hox profiles. The combinatory expression of transcription factors results in MNs assuming different class and columnar identities which are then further subdivided and guided by other molecular signaling systems.

Shh, a protein produced by the notochord, is expressed as a gradient in the ventral neural tube and acts to differentiate MN, interneuron, and floor plate cells (Jessell, 2000). Graded Shh signaling defines ventral progenitor domains through regulation of homeobox transcription factors as well as homeodomain (HD) protein expression which is then extinguished quickly in most postmitoic MNs. In the hindbrain, MNs derive from two different progenitor domains. MNs that choose ventral neural tube exit points, somatic MNs, derive from Pax6, Nkx6.1/6.2 and Olig2 expressing progenitor cells while MNs that choose dorsal exit points, visceral MNs, originate from progenitor cells that express Nkx2.2/2.9 and Nkx6.1/6.2 (Bonanomi and Pfaff, 2010; Ericson et al., 1997). One activity of Shh signaling results in repression of the homeobox gene Pax6 (Ericson et al., 1997). Nkx2.2 is only expressed in progenitors that lack Pax6 expression. Nkx2.2 represses the potential for MNs to assume a somatic identity and is in turn repressed by Pax6 activity (Briscoe et al., 1999). The expression of these HD proteins is developed in response to the Shh graded signaling system in the ventral neural tube.

Graded Shh signaling results in a patterned expression of members of the homeobox gene family in ventral progenitor cells (Agalliu et al., 2009). Unlike hindbrain MNs, all MNs from the spinal cord derive from the common ventral progenitor domain

pMN (Bonanomi and Pfaff, 2010). The combinatorial activities of Nkx6.1, Nkx2.2, and Irx3 confine MN generation to the pMN domain (Jessell, 2000). Nkx2.2 and Irx3 act to restrict Nkx6.1 but in the absence of their activity, Nkx6.1 promotes the expression of MNR2, a HD protein. MNR2 activates the expression of HB9, Isl1/2, Lim3, and other downstream transcription factors that specify MN subtype identities (Jessell, 2000). The role of Shh signaling in initiating homeobox gene expression in later MN development ultimately directs aspects such as receptor expression by certain MN subdivisions. These different combinations of receptors that are expressed allows MNs to respond to specific guidance cues in the environment that direct trajectory.

Wnt signaling activities have also been shown to have an early role in the specification of hindbrain and spinal cord progenitor cell identities. Wnt signaling serves to define cells of spinal cord character and the combined activities of Wnt and retinoic acid signaling are enough to promote cells of caudal hindbrain character (Nordström et al., 2006). The initial role of Wnt signaling in specifying progenitor cell identities is refined later on by the activities of retinoid and fibroblast growth factor (FGF) signaling. The combinatory activities of these three signaling systems induce Cdx and Hox gene expression profiles that serve as markers of the rostrocaudal position of ventral and dorsal MN generation (Nordström et al., 2006). These Hox profiles are then important in establishing the columnar organization and pool-specific identities of MNs that then determine axon projection patterns and targets.

Columnar Organization of Motor Neurons

MNs are further subdivided based on other shared qualities such as the cell type they innervate or pool organization within the spinal cord. Along the rostrocaudal axis of the spinal cord, MNs undergo columnar organization. These columnar subdivisions are determined by the activities of Hox6, Hox9, and Hox10 proteins that are distributed along the rostrocaudal spinal cord axis (Dasen et al., 2005). The spatial organization of Hox proteins is regulated by graded FGF signaling and the patterned expression of Hox proteins is controlled by Cdx homeobox gene family members (Nordström et al., 2006). Similar to graded Shh activity in the dorsoventral axis of the ventral neural tube, the spatial distribution of Hox transcriptional family members operate in the rostrocaudal axis of the spinal cord to control MN projection patterns (Liu et al., 2001). This control can most likely be attributed to differential expression of guidance receptors and activation of signaling systems.

The combined as well as individual activities of retinoids, Gdf11, and FGFs are involved in establishing the Hox-c profiles of spinal MNs (Liu et al., 2001). FGFs regulate signals from the Hensen's Node that initiate the rostrocaudal pattern of Hox-c expression by MNs. FGF signaling is in turn mediated by Cdx genes. In cervical levels of the spinal cord, retinoid signaling acts in tandem with FGF signaling in order to induce complete Hox-c profiles (Liu et al., 2001). Similarly, Gdf11 refines FGF signaling activities at rostral and thoracic lumbar spinal cord levels. Hox proteins can then interact with one another as repressors and as activators of downstream targets to define MN columnar identities.

Functionally similar MNs undergo columnar organization within the spinal cord. Neurons in the lateral motor column (LMC) grow to the limb, neurons in the medial motor column (MMC) grow to axial targets, hypaxial motor column (HMC) cells grow to body wall musculature, and preganglionic motor column (PGC) cells grow to sympathetic ganglia. HMC and LMC cells select a ventral pathway upon entering the periphery while MMC cells turn dorsally and PGC cells make a ventral turn (Bonanomi and Pfaff, 2010). Within the MMC and LMC, neurons are further characterized as medial or lateral based on their dorsal or ventral trajectory or on their location in the spinal cord.

Projection Out of the Neural Tube

The next major pathfinding choice MNs undertake is the choice to project towards ventral or dorsal exit points. MNs that extend ventrally (vMNs) consist of most spinal MNs and somatic-classes of cranial MNs (Bonanomi and Pfaff, 2010). MNs that extend dorsally (dMNs) are found mostly in the hindbrain. Both sets of MNs stem from different progenitor domains. The expression of different transcription factors by progenitor cells specifies the identities of these MN subtypes. vMNs originate from pMN domain progenitor cells that express Pax6, Lhx3, and Lhx4 (Lieberam et al., 2005). dMNs originate from p3 domain progenitor cells and express Nkx2.2/2.9 and Phox2a/b. The expression of these transcription factors activates downstream signaling systems that guide axon trajectories.

Postmitotic patterned expression of LIM-HD transcription factors and HD proteins further specifies MN identities. vMNs are characterized by a LIM code comprised of Isl1, Isl2, and Lhx3/4 while dMNs lack Lhx3/4 expression during

development. Isl1 and HB9, a LIM-HD factor and HD protein respectively, are some of the earliest expressed genes in postmitotic spinal MNs (Liang et al., 2011). Expression of the LIM-HD factor Isl1 is initially required for all MN generation and is thought to have a role in repressing the expression of Lhx3 (Jessell 2000; Liang et al., 2011). Mice that are deficient in HB9 show aberrant motor columns and errors in axonal trajectories as well as abnormal numbers of V2 interneurons (Liang et al., 2011). HB9 is an important factor in MN identity through its role in fate specification of V2 interneurons and MNs. HB9 is expressed selectively by vMNs while Phox2a/b is selectively expressed by dMNs (Lieberam et al., 2005). The selective expression of these proteins aids in establishing vMN and dMN identity.

Expression of Lhx3/4 is a key factor in specifying vMN and dMN fate. While all vMNs transiently express Lhx3/4 during their last progenitor cell division, only cells from the medial portion of the medial motor column (MMCm) continue expression of Lhx3/4 later in development (Sharma et al., 1998). In the other vMN subtypes, Lhx3/4 is down-regulated as the neurons separate into columns and project their axons ventrally. In Lhx3/4 double knock-out mice, vMNs assume properties characteristic of dMNs, showing Lhx3/4 expression to be essential for MNs to project ventrally (Sharma et al., 1998).

Spinal accessory MNs (SACMNs), a subset of dMNs, are found at cervical levels of the spinal cord and innervate the sternocleidomastoid and trapezius muscles (Dillon et al., 2005). SACMNs project axons dorsally and exit the CNS through the later exit point (LEP) before assembling into the spinal accessory nerve. During development, *Gli2* was found to be necessary for SACMN axon outgrowth, Netrin-1 and DCC were required for

the dorsal projection of SACMN axons toward the LEP, and Nkx2.9 was required for the exit of SACMN axons through the LEP (Dillon et al., 2005). Netrin-1 was thought to act through DCC to repel SACMNs away from the ventral midline. Regulation of Robo2-Slit signaling by Nkx2.9 controls the exit of SACMN axons through the LEP (Bravo-Ambrosio et al., 2012). In Nkx2.9 null mice, Robo2 is downregulated and SACMN axons are unable to project out of the CNS while vMN outgrowth is not altered. SACMN axon pathfinding itself is not disturbed in Nkx2.9 null mice although axons are unable to leave through the LEP and instead form the spinal accessory nerve within the spinal cord. The relationship between Robo2-Slit signaling and Nkx2.9 expression suggests Robo2 as a downstream effector of Nkx2.9 (Bravo-Ambrosio et al., 2012). The Robo2 associated ligands Slit1 and Slit2 have been shown to be involved with SACMN axon exit through the LEP as well as through chemoattraction.

Upon leaving the neural tube, vMN and dMNs differ in their relationship with sensory ganglia and in their axon trajectories. vMNs avoid sensory ganglia, resulting in a path that is clear of already established axonal tracts (Lieberam et al., 2005). In contrast, dMNs tend to invade sensory ganglia upon projecting out of the neural tube. These precise axon trajectory choices may be controlled by Cxcl12-Cxcr4 signaling, a G protein-coupled receptor (GPCR) signaling system. Cxcr4 is a chemokine receptor that is activated by the soluble ligand Cxcl12. The activation of Cxcr4 results in changes in growth cone motility by attenuating the actions of chemorepellents. Cxcr4 has been found to be expressed by vMNs temporarily during development and results in the evasion of vMNs from sensory ganglia in the periphery (Lieberam et al., 2005). Cxcl12 is expressed at high levels by mesenchymal cells near the spinal cord and caudal hindbrain,

where vMNs are found. Disruption of Cxcl12-Cxcr4 signaling results in the invasion of sensory ganglia by vMNs (Lieberam et al., 2005). The distinctions between vMN and dMN axonal trajectories may be explained through the actions of Cxcl12-Cxcr4 signaling in the neural tube (Lieberam et al., 2005). A lack of Cxcr4 expression by dMNs would result in response to repellents expressed by the ventral mesenchyme and floor plate, causing dMN axons to be deflected dorsally away from the floor plate. Expression of Cxcr4 by vMNs would result in an insensitivity to repellents from the ventral mesenchyme and floor plate and thus an extension of axons ventrally.

The floor plate has been identified as a major source of guidance cues. Axons respond differently to these cues based on their identity and receptor expression. Guidance cues either inhibit or promote the growth of axons and can act over short or long ranges as membrane-attached proteins or as diffusible molecules (Tzarfati-Majar et al., 2001). Growing axons are either guided towards, away from, or along the floor plate during development. Repellents and attractants can act together to guide axons towards certain targets and can have very different effects on various neuronal populations. Commissural axons, for example, grow along the floor plate before crossing it and continuing development while motor axons never cross the floor plate. The guidance cues expressed by the floor plate control axon outgrowth and growth cone dynamics during early MN development.

The ability of the floor plate to have dissimilar effects on neuronal populations is in part due to bipotential guidance molecules (Tzarfati-Majar et al., 2001). Many guidance cues are expressed by the floor plate and have the ability to either attract or repel axons based on the neuron type. Netrin-1 acts as an attractant for commissural

axons but repels certain hindbrain and midbrain MNs and Slit-2 acts as a repellent for MNs. F-spondin has been shown to be a contact-dependent repellent guidance cue for MNs but also have a role in aiding commissural neuron outgrowth (Tzarfati-Majar et al., 2001). Growth cone collapse is induced when F-spondin comes in contact with growing MNs. Since F-spondin is found to be expressed outside of the floor plate, motor axons may alter their sensitivity to F-spondin and become unresponsive or attracted to the guidance cue in later development (Tzarfati-Majar et al., 2001). Bipotential guidance molecules allow for the different trajectories exhibited by the various neuron classes that develop within the neural tube.

The three main classes of hindbrain MNs consist of somatic motor (SM) neurons, brachiomotor (BM) neurons, and visceral motor (VM) neurons (Guthrie and Pini, 1995). All three classes of MNs project their axons ipsilaterally and are initially directed away from the midline regardless of whether they choose a dorsal or ventral path out of the neural tube. This repulsion from the midline is due to the chemorepulsive effects of the floor plate. When a grafted floor plate was placed in the path of developing MNs, axons were deflected from their normal trajectories and instead took a convoluted path to reach their target (Guthrie and Pini, 1995). The response of all three classes of hindbrain neurons to the chemorepulsive effects of the floor plate mark this strip of non-neuronal cells as an important source of cues during the molecular guidance of MN development.

Boundary Cap Cells Prevent Migration of Cell Bodies

Developing spinal MNs project axons into the peripheral nervous system (PNS) while their cell bodies reside within the CNS. MN soma do not follow the same trajectory

as their axons to targets found in the periphery and instead continue to remain in the ventral spinal cord. The restraint of cell body migration during axonal projection is due to repellent signals expressed by neural crest derivatives called boundary cap (BC) cells that are located at motor exit points (MEPs) (Bron et al., 2007). BC cells regulate MN cell body migration through plexin and neuropilin interactions with semaphorins, specifically Npn-2, Plexin-A2, and Sema6A. BC cell expressed Sema6A as well as other semaphorin ligands act as repellents that help to restrict MN soma to the spinal cord as their axons initially emerge from the MEP into the periphery.

Ablation of BC cells causes MN somata to migrate out of the ventral spinal cord and follow their axons into the periphery (Vermeren et al., 2003). Removal of BC cells does not change overall motor axon growth, suggesting that axon pathfinding into the periphery occurs independently of BC cells found at the MEP and that these neural crest derivatives are not necessary for motor axons to find spinal cord exit points. In the absence of BC cells, MNs follow their axons as they exit the spinal cord, suggesting that a developmental system may exist that connects somata migration to axon projection (Vermeren et al., 2003). The presence of repellent factors by BC cells prevents neuronal cell bodies from following their axons into the periphery. The effects of neural crest derivatives are limited to MN cell bodies as motor axon trajectories into the periphery develop independently of BC cells.

Neuropilins act along with members of the Plexin-A family in order to confer signals. MNs require Npn-2 and Plexin-A2 receptor complexes and the semaphorin ligand Sema6A is expressed by ventral and dorsal BC cells (Bron et al., 2007). Sema6A helps to maintain MN soma position in the ventral spinal cord as axons project into the

periphery. A family of cytosolic signaling proteins that can directly interact with Plexin-A receptors called MICALs have been shown to be important mediators of semaphorin-plexin signaling. Studies using a member of the MICAL family known as MICAL3 have revealed the importance of semaphorin-plexin signaling in maintaining MN soma position in the ventral spinal cord. Loss-of-function studies show MICAL3 loss-of-function to be the most severe followed by Plexin-A2 and then Npn-2 loss-of-function (Bron et al., 2007). These differences in severity suggest the existence of a signaling hierarchy that begins with semaphorin interaction with neuropilin and plexin receptor complexes that produce signals which then converge on an intermediate, MICAL3 in this case. Semaphorins expressed by BC cells cause changes in MN cell bodies that results in their migration arrest and prevents their translocation out of the spinal cord with their axons. Since Sema6A and Npn-2 have not been observed to interact with one another, it has been suggested that at least two different semaphorin-neuropilin signaling pathways work together to facilitate BC cell prevention of MN somata migration (Bron et al., 2007).

Peripheral Nervous System Segmental Patterning

After projecting out of the ventral spinal cord, spinal motor axons migrate into the periphery through the sclerotome. Motor axons and neural crest cells preferentially project through the anterior-half of somites (Kuan et al., 2004; Bonanomi and Pfaff, 2010). The choice to project into the anterior over the posterior half-sclerotome is in part due to attractive cues expressed by the anterior portion and repellent molecules expressed by posterior half-sclerotome cells. Motor axons undergo contact-repulsion when

interacting with posterior cells while contact with anterior half-sclerotome cells enhance growth cone activity in motor axons (Kuan et al., 2004). Motor axons still assume their preferential projection through the anterior half-sclerotome even when the sclerotome is rotated in experiments (Fredette et al., 1996). Numerous signaling systems have been implicated in the repulsive activity produced by the posterior-half of the somite. Treatment of neural crest cells and motor axons with soluble ephrin-B1 ligands results in abnormal migration of crest cells but no outstanding changes in motor axon segmentation (Kuan et al., 2004). The different reactions of migrating neural crest cells and motor axons suggests that there may be separate molecular cues and guidance systems in place to direct growth in both populations or that there may be redundant signaling systems in place. Other repulsive signals and molecular systems implicated in motor axon outgrowth through the sclerotome are semaphorins and neuropilins, PNA-binding glycoproteins, F-spondin, and T-cadherin (Kuan et al., 2004; Bonanomi and Pfaff, 2010).

T-cadherin is expressed in the posterior half-sclerotome and is capable of inhibiting motor axon growth in vitro (Fredette et al., 1996; Kuan et al., 2004). T-cadherin has been implicated in the control of ipsilateral projection of motor axons from the spinal cord. T-cadherin acts through inhibition of stage-specific axon outgrowth in developing MNs and has been specifically implicated to play a part in the creation of the motor axon-hindlimb trajectories (Fredette et al., 1996). Expression of T-cadherin is limited to areas that are avoided by motor axons during growth and the development of lumbosacral MN trajectories hindlimb mesenchyme. T-cadherin interferes with motor axon outgrowth through repulsion of growth cones but does not arrest motor axon growth

completely. This signal to change projection patterns results in pathfinding motor axons moving away from the posterior half-sclerotome and into the anterior portion.

Few chemoattractants have been identified in the activity of the anterior half-sclerotome although their role in the guidance of motor axons through somites has been confirmed. Motor axons have been shown to project towards the closest accessible anterior half-sclerotome upon exiting the ventral neural tube near the posterior half-sclerotome (Kuan et al., 2004). Dorsal anterior half-sclerotome has been demonstrated to be chemoattractive for developing motor axons and one major chemoattractant credited for this activity has been hepatocyte growth factor/scatter factor (HGF/SF). HGF/SF is a growth factor that serves as a limb mesenchyme-derived as well as a suspected muscle-derived chemoattractant for developing motor axons (Ebens et al., 1996). HGF/SF acts through the receptor c-Met and has also been shown to function as a spinal MN survival factor. HGF/SF serves as a chemoattractant for motor axons in the sclerotome and is also important in the attractive activities of limb mesenchyme.

MMC Neuronal Development

MMC neurons project to axial targets and are the only MN subtype to expand all rostrocaudal levels of the spinal cord. MMC neuronal fate is largely controlled by postmitotic expression of Lhx3/4. The expression of these LIM-HD proteins is promoted by a ventral^{high}-dorsal^{low} Wnt4/5 signaling gradient (Agalliu et al., 2009). Wnt4/5 proteins are expressed in the ventral spinal cord and promote Lhx3/4 expression during development. In the absence of Wnt4/5 signaling, MMC neurons at thoracic levels of the spinal cord experienced a switch to HMC fate (Agalliu et al., 2009). Postmitotic Lhx3/4

expression, controlled by Wnt4/5 signaling, leads to generic MNs assuming an MMC identity within the spinal cord.

MMC neurons are divided into two major classes: MMCl neurons which are found at thoracic levels of the spinal cord and project to body wall muscles and MMCm neurons which are found at all rostrocaudal levels and project to axial muscles. MMCm axons project along the ventral root before making a dorsal turn to innervate the dermomyotome (Shirasaki et al., 2006). This guidance is controlled by fibroblast growth factor (FGF) released by the dermomyotome. FGF is one of the few chemoattractants identified in MN development. FGF acts as a long-range chemoattractant for MMCm neurons which express the receptor FgfR1. As MMCm axons extend, they are attracted to the FGF signals acting through FgfR1 receptors and are guided towards the dermomyotome (Bonanomi and Pfaff, 2010). Expression of FgfR1 is programed by the LIM code factor Lhx3 which is expressed selectively in MMCm neurons in later development (Shirasaki et al., 2006). Expression of FgfR1 and its corresponding ligand FGF are required for MMCm axons to innervate the dermomyotome through chemoattraction.

Unlike other MN subtypes, MMCm axons experience a delay during development towards axial targets (Gallarda et al., 2008). This delay results in a close association between MMCm axons and sensory axons in the dorsal ramus pathway. Instead of forming a combined path, these two neuron types separate into isolated proximal motor-sensory pathways as a result of axon-axon interactions. The sorting of these two projection pathways was found to be caused by heterotypic contact-dependent interactions (Gallarda et al., 2008). These types of encounters resulted in retraction of

filopodia and lamellipodia as well as motor growth-cone reorientation, causing the sharply divided pathways the two trajectories follow (Gallarda et al., 2008).

MMCm axons express high levels of EphA3 and EphA4 receptors. The complimentary ligand for EphA receptors, ephrin-A, is expressed in high levels by dorsal root ganglia sensory neurons. In EphA3$^{-/-}$ and EphA4$^{-/-}$ mutants, axial MNs began to invade nearby sensory pathways, showing EphA receptor activity to be necessary for the division of motor and sensory axons (Gallarda et al., 2008). Contact dependent ephrin-A:EphA signaling acts in a repulsive manner to establish discrete peripheral afferent and efferent pathways during axon outgrowth. This trans-axonal guidance occurs between axial motor growth cones and sensory neurites and prevents efferent axons from extending into afferent pathways (Gallarda et al., 2008).

LMC Neuronal Development

LMC are formed at brachial and lumbar levels of the spinal cord and project axons that innervate limb muscles. As axons project into the periphery, they converge with several other spinal nerve axons to form the cervical plexus, brachial plexus, lumbar plexus, and sacral plexus (Bonanomi and Pfaff, 2010). After pausing in this plexus region, LMC neurons select a dorsal or ventral trajectory into the limb bud. Axons that project dorsally derive from the lateral portion of the LMC (LMCl) while axons that project ventrally derive from the medial portion of the LMC (LMCm).

Both LMCl and LMCm neurons develop from progenitor cells that originate from the same dorsoventral and rostrocaudal positions (Jessell, 2000). The main difference between these two populations is their birthdate. LMC somata migrate to the ventral horn

of the spinal cord early in development (Palmesino et al., 2010). LMCm MNs leave the cell cycle before LMCl neurons and begin migration first. LMCl neurons then proceed to migrate past the earlier-born LMCm neurons to their final settling position. Reelin, an extracellular matrix protein that induces the phosphorylation of Dab1, has been found to be an essential signal in determining the location of LMC neuron somata in the ventral spinal cord (Palmesino et al., 2010). Dab1 is an intracellular adaptor protein that is required for the migration of LMC neurons and motor pool positions in the spinal cord. The Foxp1 and the LIM-HD protein Lhx1 act in conjunction to control Dab1 expression. Foxp1 is thought to maintain a basal level of Dab1 expression while Lhx1 is thought to elevate LMCl Dab1 expression. (Palmesino et al., 2010). When the Reelin system is activated, Dab1is phosphorylated and degraded. This activation is thought to control LMC migration through differential expression of Dab1 by LMCm and LMCl neurons. LMCm neurons may express lower Dab1 levels and thus Dab1 may be depleted faster in LMCm neurons than in LMCl neurons, which may have higher Dab1 protein levels (Palmesino et al., 2010). This would cease Reelin signaling and thus result in a migration stop taking place sooner in LMCm neurons than LMCl neurons.

Retinoid signaling by early LMC neurons also aids in the specification of later-born LMCl neuronal fate. LMC neurons selectively express retinaldehyde dehydrogenase-2 (RALDH2), a retinoid synthetic enzyme (Dasen et al., 2005). The activation of RALDH2 expression is controlled by the transcription factor Hox6. RALDH2 expression results in LMC neurons acting as a source of retinoids. The expression of LIM-HD proteins that govern LMC identity is controlled by the exposure

of later-born LMC neurons to retinoids (Dasen et al., 2005). LIM-HD protein expression then directs the dorsoventral division of LMC axons in limb mesenchyme.

The redistribution of axons that occurs in the plexus region is largely due to cell-cell contact facilitated by cell-adhesion molecules (Bonanomi and Pfaff, 2010). The distinct axon projections LMC neurons take upon exiting the plexus region are guided by higher levels of polysialic acid (PSA) on N-CAM, a cell adhesion molecule (Tang et al., 1994). PSA is a negative regulator of L1 which is a major receptor in facilitating MN fasciculation. When PSA was removed from the plexus region during axon sorting, many projection errors were found (Tang et al., 1994). These errors disrupted the process through which axons altered their associations with one another. It was found that L1 had an important role in the heightened axon-axon fasciculation caused by the removal of PSA from the plexus region (Tang et al., 1994). Thus, the role of PSA during sorting in the plexus region is to assist with rearrangement by reducing axonal adhesion. A decrease in axon-axon fasciculation would allow axons to change their relations with their neighbors as well as their trajectories in response to other guidance cues present in the region. PSA helps to set up the trajectory LMC axons assume by aiding in the rearrangement of axons in the plexus region.

Dorsal and Ventral LMC Projections

Upon exiting the plexus region, LMCl neurons assume a dorsal projection. This dorsal trajectory is a result of ephrin-A:EphA signaling acting in parallel with GDNF/Ret signaling. The transcription factor Lhx1 defines LMCl identity by inducing the expression of EphA receptors on LMCl neurons (Luria et al., 2008). These EphA

receptors interact with ephrin-A ligands localized in the ventral limb mesenchyme and repel LMCl axons into assuming a dorsal trajectory. LMCl axons also express c-RET receptors which interact with glial cell-derived neurotrophic factor (GDNF) found in the base of the limb (Bonanomi and Pfaff, 2010; Kramer et al., 2006). Since LMCl axons express ephrin-A ligands along with EphA receptors and EphA expression is detected in dorsal limb mesenchyme, ephrin-A:EphA signaling alone is insufficient to fully describe LMCl axon dorsal projections (Kramer et al., 2006). One explanation proposed for the coexpression of ephrin-A and EphA receptors by LMCl axons is attributed to the reverse signaling properties of ephrin-As.

MNs have been shown to be capable of expressing receptors along with their associated ligands. Ephrin:Eph signaling systems have both forward and reverse capabilities which elicit opposite effects on growth cone navigation. Within the growth cones of developing LMCl MNs, ephrin-A and EphA exhibit planar segregation which prevents *cis*-interactions between the two and thus uncouples ephrin-A and EphA signaling within the same growth cone (Marquardt et al., 2005). Interactions between ephrin-A ligands and Eph-A receptors on growth cones and the respective receptors and ligands in dorsal limb mesenchyme produces opposite responses by MN growth cones. A growth cones expressing both ephrin-A and EphA will undergo collapse when coming into contact with ephrin-A-expressing cells. The same growth cone can then be attracted to cells expressing EphA receptors that stimulate axon growth due to an uncoupling of the signaling system within the growth cone itself. Lateral segregation of ephrin-A and EphA results in prevention of *cis*-interactions and permits *trans*-interactions with Eph- and ephrin-A-expressing cells (Marquardt et al., 2005). This mechanism explains the

presence of both ephrin-A ligands and EphA receptors in dorsal limb mesenchyme and coexpression of ephrin-A and EphA by LMCl neurons and offers a more detailed explanation of the role ephrin-A:EphA signaling has in LMCl guidance. Neuropilin and plexin receptor complexes have also been shown to be coexpressed with semaphorin ligands in developing spinal MNs, suggesting that reverse signaling in other systems may also play a role in MN guidance (Marquardt et al., 2005).

GDNF/Ret signaling has been shown to be an important factor in LMCl dorsal axonal trajectories (Kramer et al., 2006). When Ret signaling is disrupted, axons that normally project dorsally are instead redirected ventrally without changing overall outgrowth. Ret expression in LMCm neurons has been shown to be capable of inducing a dorsal trajectory in the hindlimb. Ret loss-of-function studies have shown Ret loss to be heightened by the removal of EphA receptors and the opposite has been shown to be true as well. This shows that Ret and EphA signaling systems act in tandem to guide LMCl axons into dorsal limb mesenchyme. Neither Ret nor EphA are required for the expression of one another but both are required for LMCl axons to assume a normal dorsal trajectory (Kramer et al., 2006). The loss of either Ret or EphA receptors results in LMCl axon trajectory errors of varying degrees and the absence of both causes misprojections that results in all LMCl axons choosing a ventral pathway. While GDNF/Ret signaling is not needed to induce EphA receptor expression directly, one possibility is that Ret is instead essential in regulating the expression of downstream mechanisms of EphA signaling such as ephexin activity (Kramer et al., 2006).

The mechanism by which EphA receptor activation leads to repulsion into dorsal limb mesenchyme has been attributed to ephexin activity. Ephexin is a member of the

guanine nucleotide exchange factors (GEFs) of the DbI family which cause increases in the exchange of GDP for GTP and the subsequent activation of Rho GTPases (Shamah et al., 2001). When EphA receptors are activated by ephrin-A ligands, ephexin activity is altered and leads to changes in Rho GTPase signaling. EphA activation increases the ability of ephexin to activate RhoA and inhibits ephexin activity toward Cdc42 and Rac1 (Shamah et al., 2001). RhoA leads to growth cone collapse while Cdc42 and Rac1 result in filopodia and lamellipodia extension. Increased RhoA activity and decreased Cdc42 and Rac1 activity result in growth cone collapse and the repulsion of LMCl neurons from ventral limb mesenchyme. Thus, by altering the activity of Rho GTPase signaling, ephexin leads to changes in growth cone dynamics.

LMCm neurons assume a ventral trajectory after leaving the plexus region. Similar to LMCl neurons, ephrin:Eph signaling has a large role in guiding LMCm neurons into ventral limb mesenchyme. The transcription factor Isl1 expression defines LMCm identity just as Lhx1 expression defines LMCl neurons. Isl1 is required for the expression of EphB receptors on LMCm neurons and ephrin-B ligands are found in the dorsal limb mesenchyme (Bonanomi and Pfaff, 2010). Ephrin-B ligands serve as repellants that guide EphB-expressing LMCm neurons into the ventral limb mesenchyme. *EphB* triple-mutant embryos exhibit LMCm axons that assume a dorsal trajectory and LMCl axons that continue their normal dorsal projection (Luria et al., 2008). While EphA expression in LMCm neurons is sufficient to promote a dorsal trajectory, EphB expression in LMCl axons is insufficient to direct axonal projections ventrally (Luria et al., 2008). These results show that the strengths of the signaling systems differ. The strength of EphB signaling is less than that of EphA signaling in LMC neuron guidance.

The strength of EphA signaling is greater than that of EphB signaling in LMC neurons. LMCm axons then proceed to project into either ventral hindlimb mesenchyme or into ventral flank (Luria et al., 2008). The choice between these two targets may be regulated by graded EphB signaling or through distinct EphB modifiers. This choice is also a result of semaphorin-neuropilin signaling.

Semaphorins are membrane and secreted proteins that can act as guidance cues. Sema3A and Sema3F in particular are known to act as MN guidance cues by acting as a repellant and causing the collapse of spinal MN growth cones (Huber et al., 2005). Semaphorins signal through receptor complexes containing plexins and either neuropilin-1 (Npn-1) or neuropilin-2 (Npn-2). Plexins aid in signal-transducing while neuropilins act as ligand-binding subunits (Huber et al., 2005). The Sema3A-Npn-1 and Sema3F-Npn-2 signaling systems have different roles in LMC axon guidance. Sema3A-Npn-1 signaling is important in the establishment of LMCl and LMCm neuron trajectories in the forelimb. Through use of a 'surround repulsion' mechanism, Sema3A directs the fasciculation of LMC axon growth into the forelimb (Huber et al., 2005). Sema3A-Npn-1 signaling also directs the timing and fidelity of these LMC axonal projections. The establishment of dorsoventral axon trajectories requires Npn-1 expression in MNs. Sema3F-Npn-2 signaling has a more specific role in MN guidance. Sema3F is found in the dorsal limb mesenchyme and Npn-2 is expressed by a subset of LMCm neurons. The repulsive interaction between Sema3F and Npn-2 guides these LMCm neurons into the ventral forelimb (Huber et al., 2005). Npn-2 expression by LMCm neurons has a role in the creation of ventral axonal trajectories to the limb. Alternatively, the Sema3A-Npn-1 and Sema3F-Npn-2 signaling systems work together in thoracic regions of the spinal cord to

maintain the bundling of MMC axons projecting towards the intercostal muscles (Huber et al., 2005).

Recent studies on the frequency of bursting activity in spinal cords have revealed the importance of a normal frequency during MN pathfinding. When bursting activity was altered via optogenetic-mediated decreases in frequency, errors in MN dorsal-ventral pathfinding were observed (Kastanenka and Landmesser, 2013). MNs were found to express lower levels of EphA4, EphB1, and PSA when bursting frequency was decreased. EphA, EphB, and PSA are important for LMCl dorsal trajectories, LMCm ventral trajectories, and the sorting of axons in the plexus region, respectively. These results show that the normal frequency of waves of activity is important for the precise dorsal-ventral pathfinding decisions LMC neurons undertake.

The dorsoventral guidance of LMC neurons upon exiting the plexus region exhibits molecular symmetry. The important role ephrin:Eph signaling plays in both LMCl and LMCm axon guidance suggests the existence of a core Eph signaling program (Luria et al., 2008). This core system would be the main basis for dorsoventral guidance and would then be modified or regulated by other signaling systems and receptors. Whether or not a core Eph signaling system exists, other signaling systems like Sema3A-Npn-1 and Sema3F-Npn-2 are necessary to ensure proper dorsoventral axon trajectories. These molecular cues act together to guide axon guidance in LMC neurons.

Motor Neuron Pool-Specific Identities

When MNs reach the base of the limb, they separate once again into distinct motor pools based on which muscle the nerves will innervate. The rostrocaudal locations

of these motor pools are mapped onto the corresponding rostrocaudal axis of the target muscles (Bonanomi and Pfaff, 2010). While many guidance molecules and signaling systems have been implicated in the development of motor pool groupings, the main actors involved in pool-specific identity within the LMC have been identified as transcription factors of the ETS family, specifically PEA3 and ER81 (Jessell, 2000; Haase et al., 2002). Other molecules that may be implicated in the development of nerve-muscle projection patterns include the guidance molecule netrin and its corresponding receptor DCC, semaphorins, cadherins, and leucine-rich repeat proteins (Bonanomi and Pfaff, 2010). This matching process may also rely on the ephrin-A:EphA signaling system.

Motor pools can be defined by their ETS and LIM-HD transcription factor profiles. Er81 and Pea3 are ETS genes whose expression is similar in functionally related spinal proprioceptive sensory and MNs (Price et al., 2002). Along with ETS gene expression, the distinct expression of type II cadherins has been linked to motor pool identification (Price et al., 2002). MNs within each motor pool all express certain cadherin genes. Motor pools found in the lumber LMC are defined by their cadherin profiles and motor pools at rostral lumbar levels of the LMC are distinguished by the expression multiple cadherin genes. These type II cadherin expression profiles are different for each motor pool and there are no two motor pools that display the same combinatorial expression of cadherin genes (Price et al., 2002). The timing of the cadherin expression profiles by LMC MNs matches that of pool-specific ETS gene expression profiles. The constraint of cadherin expression profiles to certain subdivisions of MN corresponded with the organization of MNs into distinct motor pools. The

expression of ETS family transcription factors begins at the same time as the arrival of MNs to the base of the limb (Haase et al., 2002). This suggests that the signals initiating ETS expression are derived from the limb while the signals initiating cadherin pool-specific expression profiles depend on signals from the periphery (Price et al., 2002). ETS proteins control pool-specific cadherin expression. The varying expression of type II cadherins by LMCl and LMCm MNs that characterize each of the motor pools during sorting suggests a role for cadherins in the regulation of motor pool segregation.

GDNF has been found to induce the expression of PEA3 in specific MN populations. Motor axons that extend toward the latissimus dorsi and cutaneous maximus muscles respond to GDNF present in the periphery (Bonanomi and Pfaff, 2010). This contact results in the initiation of MN branching programs. Studies of *Gdnf* $^{-/-}$ mutants show that the majority of MNs fail to express PEA3 in the absence of GDNF signaling in the periphery (Haase et al., 2002). The expression of GDNF in the brachial plexus and limb is spatially confined to the axonal paths of specific MN populations. GDNF acts through a receptor complex consisting of RET and GFRα1 and is required for PEA3 induction in MNs targeting the cutaneous maximus and latissimus dorsi muscles (Haase et al., 2002). The differential expression of ETS family transcription factors, including PEA3, define LMC pool-specific identities. GDNF induced PEA3 is required for the proper innervation of target muscles in certain motor pool populations.

Each motor pool contains MNs that are electrically coupled which is thought to regulate the phasic firing of associated MNs (Price et al., 2002). Similar to the dorsoventral guidance of LMC neurons, changes in bursting frequency have been shown to alter pool-specific pathfinding in MNs (Kastanenka and Landmesser, 2013). When

bursting frequency was increased, pool-specific pathfinding was interrupted. These errors could be due to an interference with the capability of axons to reorganize in response to molecules that guide pool-specific grouping. More specifically, increasing bursting frequency may interfere with a signaling event within growth cones that allows them to respond to target-derived cues (Kastanenka and Landmesser, 2013). Changing the frequency of bursting activity in the spinal cord has different effects on MN guidance when it is increased or decreased. When bursting frequency is decreased, dorsoventral guidance errors occur while an increase in frequency results in pool-specific pathfinding errors. The difference in pool-specific and dorsoventral pathfinding errors based on changes in frequency indicate that these decisions rely on different downstream signaling pathways (Kastanenka and Landmesser, 2013).

Phr1 is a protein associated with the microtubule cytoskeleton that has been found to be required for correct synapse formation (Bonanomi and Pfaff, 2010). Phr1 is expressed specifically within the shaft of axons during MN pathfinding and is expressed in lower levels in distal branches and growth cones (Lewcock et al., 2007). Mouse *Magellan* mutants, which displayed a truncated form of Phr1, exhibited various stalling and growth defects at major MN choice points, including those made at the limb level (Lewcock et al., 2007). *Magellan* mutants showed accurate MN cell body and columnar organization as well as normal guidance receptor expression and responses to guidance cues. Growth cones in *Magellan* mutants failed to exhibit normal development and often did not stay positioned on distal portions of the axon. Phr1 was found to aid in the stability of microtubules in the axon shaft by preventing DLK accumulation in stable axonal regions (Lewcock et al., 2007). One function of Phr1 is to prevent dynamic

cytoskeletal structures from forming in stable portions of the axon shaft. The loss of Phr1 results in microtubule destabilization and numerous trajectory errors in MNs.

Conclusion

MN development occurs in a step-wise manner starting with the induction of a general MN identity by Shh signaling. MNs then begin to be subdivided based on their LIM code, HD expression, and Hox profiles. These transcription factors serve to drive receptor expression and initiate downstream signaling systems. MNs then separate into different columns within the spinal cord according to their targets in the periphery. The guidance of these subsets of MNs then rely on different signaling systems to reach their destinations.

MNs are diversified in their cell types and targets and undergo reorganization several times throughout development based on increasingly specific similarities. While all MNs originally develop from few progenitor domains and are induced by the same signals, further guidance and signaling systems are widely varied and specific to certain subsets of MN. Many developments have been made in identifying molecular guidance systems that aid in MN pathfinding but there are still many aspects of MN guidance that remain unknown. For instance, relatively few chemoattractants have been identified in MN guidance although their role in pathfinding has been confirmed.

It is not yet fully understood how various MN subtypes can be guided through the same environment and respond to guidance molecules differently. Given that few guidance molecules have been identified in MN guidance, their role in guiding axon projections must have diverse functions. Ephrin:Eph signaling activity, for example, has

been implicated in MMCm, LMCm, and LMCl axon trajectories as well as the development of MN pool-specific identities. Further research into the guidance molecules implicated in MN development, both classic as well as newly discovered, will provide insight into how MNs are guided by such a small population of guidance molecules and how these limited signaling systems operate in determining the different trajectories of the diverse MN subdivisions.

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