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Motor neuron migration and positioning mechanism s: new roles for guidance cues

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Abstract

Motor neurons differentiate from progenitor cells and cluster as motor nuclei, settling next to the floor plate in the brain stem and spinal cord. Although precise positioning of motor neurons is critical for their functional input and output, the molecular mechanisms that guide motor neurons to their proper positions remain poorly understood. Here, we review recent evidence of motor neuron positioning mechanisms, highlighting situations in which motor neuron cell bodies can migrate, and experiments that show that their migration is regulated by axon guidance cues. The view that emerges is that motor neurons are actively trapped or restricted in static positions, as the cells balance a push in the dorsal direction by repulsive Slit/Robo cues and a pull in the ventral direction by attractive Netrin-1/DCC cues. These new functions of guidance cues are necessary fine-tuning to set up patterns of motor neurons at their proper positions in the neural tube during embryogenesis.

Keywords

motor neurons; brain stem; spinal cord; floor plate; Slit/Robo; Netrin-1/DCC

Introduction

Motor neurons (MNs) are born from progenitor cells in the ventricular zone of the brain stem and spinal cord. Progenitor cells in a narrow column are exposed to a specific concentration of Sonic hedgehog (Shh) morphogen and respond by expressing a cascade of transcriptional regulators that specifies their differentiation as MNs and clustering into motor nuclei next to the floor plate [1–3]. The clustering of MNs is important for their function, as input fibers bring their synapses into the motor nucleus, and the motor nuclei serve to coordinate the projections of motor axons to exit the central nervous system (CNS) and project to their targets in the periphery [4–6]. Moreover, these neurons are electrically

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coupled to their neighbors by gap junctions, and thus functionally related neurons are firing together in response to afferent inputs [7, 8]. Therefore, accurate positioning of MN cell bodies to cluster them in the correct location within the motor nucleus is a key step for their functional input and output.

Although the initial acquisition of MN identity and later mechanisms that guide motor axons have been examined in depth, the mechanisms in which MN cell bodies find their proper position in the neural tube have been studied in less depth. Several lines of evidence suggest that MNs can respond to guidance cues via their axons [9–12]. However, much less is known about how axon guidance cues could influence the migration and/or positioning of the MN cell bodies.

In this short review, we would like to summarize what was previously learned about MN positioning mechanisms. Then we will describe more recent evidence for new mechanisms of MN positioning, in which MN are positioned by axon guidance signals, specifically, push by Slit/Robo repulsion and pull by Netrin-1/DCC attraction.

Motor neurons have the potential to migrate

MNs derive from progenitor cells, located in a ventral region of the neural tube and settle next to the floor plate. Since their progenitors are located near the floor plate, these cells are exposed to varying concentration of Shh morphogen, which sets off a cascade of transcription factors that specifies MN identity for the cells at this position. Thus, the development pattern of MNs suggests that these cells are simply produced where they are needed, and perhaps an ability to migrate is not part of their developmental repertoire.

Although MNs generally undergo only local shifts within their nuclei, a few types of MNs do migrate, with the best known example being the facial branchiomotor neurons in the hindbrain of vertebrate embryos. The facial motor neurons undertake a unique and complex migration, in which they first project axons to their exit point, but then their cell bodies migrate tangentially to more posterior segments in the hindbrain, along a ventral position near the floor plate, and then switch to migrate dorsally within their new segments [13, 14]. For these distinct steps of facial motor neuron migration, studies of the migration mechanisms over the past couple of decades have identified important roles for Wnt, cadherin, and a number of intracellular signaling pathways in their migration. Facial motor neuron migrations are in part guided by floor plate signals, such as repellent Slit/Robo signals during their posterior migration [15]. The extreme migration ability of facial motor neurons could be viewed as a special ability that these neurons have gained to get them to their unique final locations. However, several examples have emerged of other MN types being able to migrate, at least in certain experimental conditions, suggest a broader migration potential of MNs.

It should also be noted that there are two other examples of cranial motor neurons that migrate. In the midbrain, a subset of oculomotor neurons migrate across the ventral midline to reside in the contralateral oculomotor nucleus [9], and these will be discussed further in a section below. The other example is a small group of acoustic motor neurons that similarly

cross the midline in rhombomere 4 [16], but have not been studied. Neither of these examples of MN migration are as well understood as facial motor neuron migration, but they do demonstrate MN migration elsewhere in the brain stem.

Another important line of evidence hinting at the potential broad ability of MNs to migrate involves a unique subset of neural crest cells in the spinal cord, the boundary cap cells. Along the outside of the spinal cord, these cells migrate to the ventral motor exit points and dorsal sensory entry points. Boundary cap cells act as a selective barrier to MN cell bodies while letting motor axons pass through the pial surface to the periphery. The barrier function of boundary cap cells is implicated by the experiment of ablating the boundary cap cells, which leads to emigrant MNs outside the neural tube at the ventral exit points, suggesting these neural crest cells are necessary for keeping MNs inside the neural tube [17]. An interesting implication of MN emigration is that the MN cell bodies follow their axons out into the periphery. Boundary cap cells appear to carry out their function as a barrier to MN migration through specific guidance cues. For example, repulsive Semaphorin signals from boundary cap cells prevent MN cell bodies to translocate into the periphery [17, 18]. Another recent report revealed that a new Netrin family, Netrin5 is expressed by boundary cap cells and Netrin5/DCC signals also prevent MN migration out of the neural tube [19]. Together, these reports of the barrier function of boundary cap cells in the spinal cord strongly implicate that MNs have a migratory ability.

Interestingly, a recent study showed that the transcription factors, *Islet1* and *Islet2* restrain MN cell bodies within the spinal cord by regulation of Slit/Robo and Sema/Nrp signaling [20]. Specifically, in *Islet* mutant mice, many MNs migrate out the neural tube regardless of their subtype. Also, the emigrant MNs in the mutants showed downregulation of *Neuropilin1* and *Slit2* transcripts, suggesting that these genes could be the target genes of the Islet proteins. Indeed, Robo or Slit mutations in mice and knockdown of Nrp1 or Slit2 in chick embryos caused MNs migrate to the periphery. Together, these findings suggest that Islets are involved in maintaining MNs inside the spinal cord by regulation of these repulsive signals. Interestingly, in the *Islet* or *Robo1/2* double mutants, MNs migrate out the spinal cord at embryonic day 9.75 (E9.75) when the boundary cap cells have not established at the ventral exit points, implying that these *Islet*-*Slit* mediated signals are boundary cap cell-independent, and are required for keeping MNs inside the neural tube before these neural crest cells function as a barrier [20]. There are therefore at least two separate levels of inhibitory checkpoints to ensure that MNs remain inside of the spinal cord.

Together, these examples of migration suggest that MNs have a general migratory ability, but this normally remains suppressed, except in a few unusual populations of cranial motor neurons which can overcome this suppression. The recent observations of MN migration in response to particular experimental conditions suggests that the static view of MN migration needs to be revised.

Motor neurons migrate along an axon or axon-like leading process

For MN cell bodies to overtly migrate, or to undertake more subtle shifts to new positions within motor nuclei, the cell bodies themselves must have a way to migrate. In surveying the

examples known so far, the MN cell body migration is led by an axon or axon-like leading process. Axon-like leading processes are formed by both oculomotor neurons migrating across the midline of the midbrain [9, 10], and by facial branchiomotor neurons migrating posteriorly in the hindbrain [11, 13]. A study showed that the actin-binding cytoplasmic protein, Drebrin is necessary for the migration of oculomotor neuron by regulating the formation of the leading process and controlling the orientation of the process [21]. In the study, they found that when Drebrin is missing, the leading processes are not formed and thus oculomotor neurons fail to migrate [21], suggesting the essential role of the correct formation of the leading process in MN migration.

Experimental conditions that induce ectopic MN migration also involve a similar sequence, starting with axon projections, then the cell bodies migrating by translocating along those axons. In the case of disrupting boundary cap cells or their cues, motor axons exit normally, but then MN cell bodies stream out after, following their axons to emigrate out of the neural tube to stream along within the motor nerve roots [17–19]. Thus, the emigrating MN cell bodies did not exit independently, but followed their axons out. In this scenario, the motor exit point and the associated boundary cap cells normally act as a selective filter, allowing motor axons out but not MN cell bodies. When this selectivity is lost, MNs reveal their ability to migrate along their axons. Interestingly, if motor axons are redirected into abnormal pathways within the CNS, MNs can similarly follow their axons. We noticed in our studies of MNs shifting into the floor plate in *Slit* and *Robo* mutant mouse embryos that many of the ectopically placed cell bodies were clearly associated with motor axons growing long distances longitudinally within the floor plate [12]. Similarly, the premature migration of oculomotor neurons across the midbrain midline in *Slit* and *Robo* mutants is preceded by secondary formation of axon-like leading processes that cross the floor plate [9]. Therefore, in each case of MN migration, it appears that MN cell bodies do not require an independent mechanism to migrate, but have a tendency to translocate along their axons, unless prevented by a selective barrier such as the motor exit point and the associated boundary cap cells.

The tendency of MNs to follow their axons might also explain how MNs make their more subtle but specific shifts to form their topographic organization of motor pools within the spinal motor nuclei. The topographic positioning of motor pools is not yet well understood in terms of molecular mechanisms and potential guidance cues. Initial studies have implicated Cadherin-catenin signaling as important for shifting motor neurons to their topographically appropriate positions [22, 23]. However, while it seems likely that other guidance cues in the ventral spinal cord also influence the topographic organization of motor pools, these cues may not act on the MN cell bodies but on their outgrowing processes. We speculate that motor dendrite fibers could lead the path for topographic migration. The rapid formation of motor axons and exit from the spinal cord is followed soon after by formation of dendrites, with many MNs having extensive projections infiltrating dorsally and medially across the nucleus [24]. The size and directionality of this dendritic tree growth is likely regulated by additional external cues, possibly other diffusible or substrate cues to form specific connections with the diverse input synapses carrying positive and negative motor signals. As the dendritic branches make their own organized projections, the dendritic neurites could provide a substrate for directed MN cell body shifts into topographically appropriate positions. The cell bodies would subsequently be in position to receive axo-

somatic synapses, as well as become directly electrically connected by gap junctions to their motor pool neighbors. A prediction of this proposed dendrite-mediated migration mechanism is that treatments that inhibit or misdirect motor dendrites should also block or alter motor pool topography.

The roles of axon guidance signals in controlling neuron migration

Since newly born MNs settle next to the floor plate and these cells have the tendency to follow their axons, floor plate guidance cues have the potential to keep MNs to their proper position. Indeed, the major floor plate-derived repellents, Slit family and their Robo receptors guide migration of diverse neuron types [25, 26]. For example, Slit/Robo repulsion guides neuroblasts during the long migratory route from the subventricular zone to the olfactory bulb [26]. In the mouse hindbrain, *in vitro* and *in vivo* studies show that migrating precerebellar neurons use Slit/Robo signals to find their proper position [27, 28]. Similarly, in the midbrain, Slit/Robo signals inhibit premature migration of oculomotor neurons [9]. It has been reported that Slit/Robo signals control neuronal migration by regulating cell polarity, mediated by local Ca^{2+} transients, redistribution of active RhoA, and/or centrosome positioning [29, 30].

As for counteracting attractive signals, Netrin-1 and its attractive receptor DCC are involved in many neuronal populations for axon guidance and neuron migration [31–34]. For instance, *in vivo* and *in vitro* studies showed that Netrin-1 is required for oligodendroglial migration into the olfactory bulb [35]. Also, Netrin-1 plays a role in guiding migrating pontine neurons by attracting their leading processes [32]. In addition, *in vitro* culture assay shows that Netrin-1 attracts tangentially migrating of lot cells. They also reveal abnormal distribution of lot cells and errors in lateral olfactory tract (LOT) projection in Netrin-1 or DCC mutants, suggesting Netrin-1/DCC signals guide migration of lot cells. It has been reported that Netrin-1 induced precerebellar neuron migration requires phosphorylation of the microtubule-associated protein, MAP1B by the activation of the serine/threonine kinases cyclin-dependent kinase 5 (CDK5) and glycogen synthase kinase 3 (GSK3) [36]. Indeed, MAP1B mutant mice show migration defects in the pontine nuclei, similar to the phenotypes reported in Netrin-1 or DCC mutants [36, 37].

Together, these findings set up a strong rationale for the idea that axon guidance signals are involved in various types of neuron migration and thus strongly imply the potential role of guidance signals, especially the major floor plate repellants, Slits and the major attractant, Netrin-1 in controlling MN migration and position in the neural tube.

Slit/Robo and Netrin-1/DCC signals set the proper position of motor neurons

Using mouse embryos with mutations in the guidance signals, we recently reported the roles of Slit and Netrin-1 signals in positioning MNs in the brain stem and spinal cord [12]. First, we revealed that MNs expressed the Robo1 and Robo2 receptors by using reporter inserts for the Robo1 and Robo2 mutant alleles [38], and we performed immunostaining of dissociated MNs with anti-Robo1 and anti-Robo2 antibodies, which clearly showed that

Robo receptors were expressed on the cell membrane and internal membrane structures of MN cell bodies. Together, the expression patterns of Robo receptors evidently imply that these cells are potentially responsive to repulsive Slit signals. In addition, we observed that MNs entered the floor plate in the hindbrain and spinal cord when the secreted Slit proteins or their Robo receptors are absent. Conversely, in the brain stem and spinal cord, MNs also expressed the DCC receptors and shifted dorsally when the ventral Netrin-1/DCC attractant signal is disrupted. These opposing shifts between Slit/Robo and Netrin-1/DCC mutants suggest a positioning mechanism in which actively migrating MNs are held in balance between the repellent Slit/Robo and the attractive Netrin-1/DCC signals (Figure 1).

Using mouse genetic approaches, we demonstrated that Slit2 is the main floor plate repellent and Robo2 is the primary mediator of Slit repulsion in order to maintain MNs in their normal position. Double labeling with the progenitor markers, NKX6.1 or Olig2 with the MN marker, Islet-1 showed that MNs are generated from normal progenitor columns, then migrate tangentially into the floor plate in Robo mutants. Using *Isl1^{MN}-GFP* mice at the earliest stage of migration, E9.5 we observed that mis-positioned MN cell bodies had a bipolar shape, with axon-like processes leading into the midline in Robo1/2 double mutants. The first ectopic MNs appear to pioneer an independent pathway into the floor plate, because the ectopic MNs enter the floor plate before any commissural axons, which begin to appear by E10.5, or any other β III-tubulin⁺ neuron fibers. Together, these findings suggest that, when Robos are missing, motor axons are mis-directed to projecting into the floor plate, and MNs migrate tangentially into the floor plate. Interestingly, this also supports a previous report in which Slit signals influence neuron polarity [29, 30].

Because a significant number of MNs with bipolar morphology were found in the floor plate in Robo1/2 double mutants, we tested whether these MNs were still able to project axons to their normal exit points. Using *Robo1^{+/-};2^{-/-}; Islet-1^{MN}-GFP-F* embryos and retrograde diI axon tracing, we demonstrated that axons from the mis-positioned cell bodies projected within the floor plate instead of finding their normal exit point in Robo mutants. However, there were no axon projections within the floor plate in wild type embryos, suggesting the vital role of Slit/Robo repulsion in guiding MN cell bodies and their axons into their normal paths. Similarly, Islet-1⁺ motor columns shifted dorsally from normal progenitor columns in Netrin-1 mutants, suggesting that MNs differentiate normally from progenitor cells and then migrate dorsally in the absence of attractive Netrin-1/DCC signals. Together, these findings suggest that attractive Netrin-1/DCC signals are required for setting the proper position of MNs in the neural tube. Future studies will be necessary to determine how Slits and Netrin-1 affect MN migratory mechanism possibly by exploring centrosome position via *in vivo* and *in vitro* approaches to determine whether Slit/Netrin signals control cell polarity and axogenesis, or whether the guidance cues are restricted to steering the axons after they emerge. Furthermore, it remains to be investigated the intracellular mechanisms for how MNs integrate the opposing Slits and Netrin-1 signals to find their precise position in the neural tube.

The consequences of abnormal MN migration might include a partial depletion of motor pools, abnormal motor synapses in the CNS, or abnormal exits. However, our observations suggest that many mis-positioned MNs disappear at later embryonic stages, possibly

because they die, migrate away, or turn off MN markers. Additionally, examining the functional consequences of mis-positioned MNs is not possible because the Robo or Slit knockout alleles are perinatal lethal as homozygotes. Future experiments using MN-specific Robo knockouts could overcome the lethality of these mutants and allow tests for functional significance in specific motor systems.

Overall, our evidence shows a new mechanism of MN positioning in the neural tube, in which MN are actively migrating cells, and are normally trapped in a static position by Slit/Robo repulsion and Netrin-1/DCC attraction.

MN migration across the midline utilize Slit/Robo signals

MNs that migrate across the floor plate provide an interesting case study into how MN utilize floor plate-derived guidance cues. There are two populations of MNs that migrate across the midline; vestibulo-acoustic efferent neurons and a subset of oculomotor neurons. Both populations first project an axon which exits normally toward peripheral targets, but then the cell bodies migrate in the opposite direction, trailing their primary axon to forming a commissure across the ventral midline early in development. Therefore, MN cell bodies are located contralateral to their muscle target. Migrating vestibulo-acoustic neurons differentiate adjacent to facial motor neurons and are located ventral to the facial nuclei prior to migration [39]. Likewise, migratory oculomotor neurons originate from the ventral-most region of the oculomotor nucleus. This ventral location suggests that these MNs in particular may utilize floor-plate derived cues to guide their migration. Investigation into the role floor plate cues play in guiding contralateral MN migration has been examined in the mouse oculomotor system but has not been explored in vestibulo-acoustic neural migration.

In the midbrain, both Slit and Netrin guidance cues are expressed at the floor plate in the midbrain [40]. We found that oculomotor neurons express Slit receptors, and are sensitive to Slit repulsion from the floor plate to maintain their ipsilateral position prior to migration. Loss of Slit signaling in mutant mice results in a subset of oculomotor neurons migrating across the ventral floor plate 3 days prior to normal migration (neurons migrate on E10.5 instead of E13.5).

Normal migration of oculomotor neurons is preceded by the extension of a leading process that orients toward the ventral floor plate and in some cases reaches the contralateral oculomotor nucleus [9, 10]. As mentioned above, the leading process is dependent upon the expression of an Drebrin, an actin-binding protein [21]. Oculomotor cell bodies translocate within this leading process to reach the contralateral nucleus [9]. In Slit and Robo mutant mice, premature migration also includes the extension of leading processes. However, the leading processes are disorganized as they attempt to project across the midline. Therefore, it is likely that the leading processes rely on Slit signals to guide navigation across the floor plate, much like navigating commissural axons.

This study raises interesting questions into what is unique to the E10.5 period in which oculomotor neurons are capable of migration and sensitive to Slit signals, and whether this critical period exists in other non-migratory MNs. For oculomotor neurons, this timing

mechanism may be due to the integration of multiple cues. For example, Shh expression is localized to the floor plate in the midbrain on E10.5, but fans laterally as development proceeds and is absent in the floor plate by E12.5 [41]. Transient expression of guidance cues like Shh, and others, may create critical periods which regulate when MNs are sensitive to specific cues.

Other regulators of motor neuron positioning

In addition to classical axon guidance cues, there are clearly many other regulatory factors that are needed to confer migration ability, and to guide and modulate this migration. Previous studies have shown that transcription factors dictating MN specification are involved in positioning MNs at the right location. Indeed, the LIM-homeodomain transcription factors, Isl2 and Lhx1 are critical for the location of visceral and limb-innervating MNs, respectively, and the ETS class transcription factors, PEA3 and ER81 are required to form specific motor pools in the spinal cord [42–44]. Additional mechanisms that act in clustering and positioning MNs have been described in the spinal cord. For instance, cadherin-catenin signaling mediate cell-to cell adhesion of spinal motor neurons [22]. Also, Reelin signaling determine the proper position of MNs in the spinal lateral motor column [44]. Further research is needed to define the molecular mechanisms of how other types of MNs find their correct position in the neural tube.

Future directions and applications

Several lines of evidence investigating the combined action of guidance cues show that various ways of signal integration of multiple signals are involved in guiding the correct path of diverse types of axons [45, 46]. For instance, for commissure axon guidance in the spinal cord, the Slit-gated silencing of Netrin attraction is the predominant model [47], yet motor axons evidently can simultaneously use Slits and Netrin-1 as opposing sets of cues. This Slit/Netrin balance conflicts with the prior evidence for Netrin-1 silencing, but which should be noted came mainly from *in vitro* experiments on individual cultured axons. Another set of evidence inconsistent with silencing has come from a study of hindbrain commissural axons, which were shown to continue to use Netrin-1 attraction even after crossing the floor plate [48].

It has become increasingly evident that Netrin and Slit signals can interact in diverse ways in different types of neurons. For example, in thalamocortical axons Slits act through a co-receptor complex to switch on Netrin attraction, while the opposite effect has been reported in the developing corpus callosum where Netrin attenuates Slit repulsion [49–51]. An example that may be similar to the mechanism that we have uncovered for MNs positioning is the parallel independent Netrin and Slit guidance of fly commissural axons [52]. Using *in vivo* and *in vitro* experiments, we have shown that simultaneous Netrin-1/DCC and Slit/Robo signals guide pioneer longitudinal axons in the mouse hindbrain, providing another example of neurons that balance Netrin-1 attraction and Slit repulsion [53]. These different consequences of Slits and Netrin-1 signals in the various cases might be due to different concentrations of guidance cues, different combinations of receptors, or diverse signaling cascades involved in various types of neurons. Together, these proposed integration

mechanisms provide a powerful tuning of preventing guidance errors and imply that these mechanisms might be involved in guiding MNs at the proper position. In fact, it has been reported that other guidance signals, such as *Sema3A* and *Ephrins* are involved in spinal motor axon navigation [54, 55]. Interestingly, a recent paper reported synergistic integration of *Netrin-1* and *ephrin* in spinal MNs by forming a complex of the *Netrin* receptor, *Unc5c* and the *ephrin* receptor, *EphB2* [55]. These findings support the possibility in which multiple guidance signals might be involved in guiding MN cell bodies at their proper position in the neural tube. Thus, further research into the mechanisms of how MNs integrate multiple signals will be needed to more fully understand the positioning mechanisms of MNs in the neural tube.

Current treatment for MN diseases such as spinal cord injury (SCI) and spinal muscular atrophy (SMA) are extremely limited, emphasizing the need to develop new approaches to restore MNs and their functions. Since embryonic stem cells (ESCs) have the potential to replace diverse cell types, transplants of ESC-derived cells into the injured tissue would have the potential to restore MN functions. Indeed, the transplant of ESC-derived MN progenitor cells into injured spinal cord results in recovery of mobility in SCI animal models [56]. Furthermore, recently this treatment has begun testing in human patients. Therefore, it is fundamental to understand how normal developing neurons migrate in response to existing guidance signals within the brain stem and spinal cord. Future studies need to be tested *in vitro* whether these signals dictate accurate MN positioning and thus which guidance signals need to be controlled for ESC transplants to restore motor function in MN diseases.

Conclusion

Overall, axon guidance signals have emerged important regulators of MN positioning within the neural tube. Recent evidence implies that the clustering of MNs to form motor nuclei is not simply the outcome of where they are induced and born, but these neuron populations depend on axon guidance cues to stay within their nucleus. This positioning mechanism seems to act as an important developmental fine-tuning, possibly a backup positioning system to add another layer of protection from errors in MN specification or migration. The tendency of MN cell bodies to follow their axons appears to be a key step for the limited normal migration of cranial motor neurons, and for experimental cases of abnormal migration. Understanding how normal developing MNs migrate in response to multiple guidance signals within the neural tube will be critical for identifying essential signals to set the proper position of MNs.

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Figure 1. Axon guidance cues are required to keep motor neurons in their normal position
 After progenitor cells (PC) shift out to the mantle zone (gray arrows) and begin to differentiate as motor neurons (MN), they are kept in motor columns by Slit/Robo repulsion (red) and Netrin-1/DCC attraction (green) (from Kim et al.[12]).