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Motor neuron cell bodies are actively positioned by Slit/Robo repulsion and Netrin/DCC attraction

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ABSTRACT

Motor neurons differentiate from a ventral column of progenitors and settle in static clusters, the motor nuclei, next to the floor plate. Within these cell clusters, motor neurons receive afferent input and project their axons out to muscle targets. The molecular mechanisms that position motor neurons in the neural tube remain poorly understood. The floor plate produces several types of guidance cues with well-known roles in attracting and repelling axons, including the Slit family of chemorepellents via their Robo receptors, and Netrin1 via its DCC attractive receptor. In the present study we found that Islet1^{+} motor neuron cell bodies invaded the floor plate of Robo1/2 double mutant mouse embryos or Slit1/2/3 triple mutants. Misplaced neurons were born in their normal progenitor column, but then migrated tangentially into the ventral midline. Robo1 and 2 receptor expression in motor neurons was confirmed by reporter gene staining and anti-Robo antibody labeling. Mis-positioned motor neurons projected their axons longitudinally within the floor plate, and failed to reach their normal exit points. To test for potential counteracting ventral attractive signals, we examined Netrin-1 and DCC mutants, and found that motor neurons shifted dorsally in the hindbrain and spinal cord, suggesting that Netrin-1/DCC signaling normally attracts motor neurons closer to the floor plate. Our results show that motor neurons are actively migrating cells, and are normally trapped in a static position by Slit/Robo repulsion and Netrin-1/DCC attraction.

Introduction

Motor neurons are a prototypical example of a cell type induced at a specific position within a morphogen gradient. Because of their position near the floor plate, neural progenitors in a narrow column are exposed to a specific concentration of Sonic hedgehog morphogen and respond by expressing a cascade of transcriptional regulators that specifies their differentiation as motor neurons and clustering into motor nuclei (Briscoe and Ericson, 2001; Briscoe et al., 1999; Osterfield et al., 2003). The position of motor neuron clusters is important for the function of motor circuits, because motor neuron position influences where their axons exit the CNS toward their peripheral muscle targets (Bravo-Ambrosio and Kaprielian, 2011), which synaptic inputs they receive from sensory and other neurons (Chang and Balice-Gordon, 2000; Fritzsch et al., 1993; Song and Pfaff, 2005), and whether neighboring neurons become electrically coupled within motor pools (Brenowitz et al., 1983; Kiehn and Tresch, 2002). Motor neuron cell bodies can shift locally within their nucleus to settle into topographic order (Leber and Sanes, 1995), with these shifts regulated by Reelin and cadherin signaling (Demireva et al., 2011; Palmesino et al., 2010; Price et al., 2002; Yip et al., 2000). Therefore the functional organization of motor nuclei relies on motor neurons being precisely induced in the correct position and remaining in their local nucleus.

However, a few motor neuron populations in the brain stem do undergo substantial migrations out of their local motor nuclei. A well-known example is the facial branchiomotor population which migrates posteriorly to traverse hindbrain segments alongside the floor plate (Auclair et al., 1996; Bingham et al., 2010; Chandrasekhar, 2004; Fritzsch and Nichols, 1993; Glasco et al., 2012). Likewise, a subset of oculomotor neuron cell bodies migrates across the ventral midline of the midbrain to the contralateral nucleus (Chilton and Guthrie, 2004; Fritzsch et al., 1995; Puelles-Lopez et al., 1975). Interestingly, Semaphorin or receptor mutations cause motor neuron cell bodies to translocate out of the spinal cord (Bron et al., 2007; Vermeren et al., 2003), which suggests broader capacity of motor neurons to migrate.

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Because motor neuron cell bodies cluster close to the floor plate, guidance molecules from the floor plate could regulate the position of these cells. The Slit family of repellents, and their Robo receptors, guide migration of diverse neuron types (Kidd et al., 1999; Wu et al., 1999). However, tests of Slit/Robo functions in motor neuron position and axon guidance have given mixed results. Expression of dominant negative Robo receptors in chick hindbrain motor neurons, predicted to interfere with Slit responses, caused some motor axons and motor neuron cell bodies to shift into the floor plate, consistent with Slit/Robo repulsion (Hammond et al., 2005). However, contrasting with the chick embryo experiments, mouse embryos mutant for Robo1, Robo2, or with combined Slit1 and 2 mutations, resulted in motor axons growing into the floor plate, but not motor neuron cell bodies (Hammond et al., 2005), which was further supported by a separate analysis of Robo1/2 combined mutant mouse embryos (Bai et al., 2011). In contrast, Slit2 promotes the axon outgrowth in culture of a spinal cord accessory motor axon population (Bravo-Ambrosio et al., 2012). The discrepancies between these results leave unresolved the in vivo role of Slit/Robo signals in guiding motor neurons in hindbrain or spinal cord. As for counteracting attractive signals from the floor plate, Netrin-1 and its attractive receptor DCC are required in many neuronal populations for axon guidance and neuron migration (Culotti and Merz, 1998; Kawasaki et al., 2006; Serafini et al., 1996; Yee et al., 1999). Surprisingly, a repulsive function of Netrin-1 for motor neurons has been proposed, because cranial motor axons in vitro are repelled by Netrin-1 (Colamarino and Tessier-Lavigne, 1995; Murray et al., 2010; Varela-Echavarría et al., 1997), and this repulsive effect can be blocked by antibodies against the repulsive Netrin-1 receptor Unc5a (Murray et al., 2010). Similar to Slit perturbations, mouse Netrin-1 mutations caused a subset of cranial motor axons to grow into the floor plate, but consistent misplacement of motor neuron cell bodies into the floor plate was observed only in chick embryos following expression of a dominant negative Unc5a receptor (Murray et al., 2010). In contrast, cultures of spinal cord motor axons are normally unresponsive to Netrin-1, although a switch to attraction can be caused by perturbations of Presenilin and its proteolytic processing of the DCC receptor (Bai et al., 2011). Together, the above studies suggest that floor plate signals are important for guiding motor axons, and also in some cases for positioning their cell bodies. However, the discrepant results between genetic strategies, i.e. knockout mice, and misexpression/dominant negative strategies, i.e. in chick, leave unresolved the in vivo relevance of which floor plate guidance cues might set the position of motor neuron cell bodies, and whether these roles are positive or negative.

The present study is based on our unexpected observation that neuronal cell bodies were present in the hindbrain floor plate of early Slit and Robo mutant mouse embryos (Kim et al., 2011). This observation led us to focus on the earliest stages of motor neuron development in the brain stem and spinal cord to reevaluate the in vivo roles of Slit/Robo and Netrin1/DCC signals in positioning motor neuron cell bodies. Through a range of observations on mutant mouse embryos, we found that the normal position of motor neurons is due to migratory reactions to repulsive Slit and attractive Netrin signals.

Materials and methods

Mouse embryos

Mice were maintained and the experiments carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, by protocols approved by the University of Nevada, Reno Institutional Animal Care and Use Committee. Embryonic day 9.5 (E9.5), E10 and E10.5 embryos were obtained via uterine dissection. Wild type CD-1 mice (6–8 weeks old) were purchased from Charles River Laboratories (Wilmington, MA USA). The Robo, Slit, Netrin-1, and DCC mutant strains were gifts of Marc Tessier-Lavigne, Genentech, and Frederic Charron, ICMR, Montreal CA. Robo and Slit PCR genotyping were performed as previously described (Grieshammer et al., 2004; Plump et al., 2002). Netrin-1 and DCC genotyping were performed as previously described (Fazeli et al., 1997; Serafini et al., 1996). The Islet-1^{MN}-GFP-F strain was a gift of Samuel Pfaff (Lewcock et al., 2007), Salk Institute, and was crossed into CD1 and Robo1/2 mutant backgrounds.

Immunohistochemistry

For whole-mount immunolabeling, embryos were fixed in 4% PFA overnight and prepared by dissecting out the neural tube and washing for several hours in PBS containing 10% fetal bovine serum and 1% Triton X-100 (PBST). Primary antibodies used were rabbit anti-βIII-tubulin (Covance. 1:1000), rabbit anti-Robo1 and Robo2 (kind gift of Elke Stein, Yale, 1:10,000), mouse anti-Islet-1 (DSHB, 1:100), goat anti-DCC (Santa Cruz, 1:250), mouse anti-NKX6.1 (DSHB, 1:100), rabbit anti-Olig2 (Millipore, 1:1000), rabbit anti-Phox2b (Pattyn et al., 1997) (1:2000), and mouse anti-4C7 (DSHB, 1:1). Primary antibodies were applied in PBST for 2–3 days. Our previous characterization of the Robo1 and Robo2 antisera showed specific labeling by immunofluorescence that was lost in homozygous mutants (Kim et al., 2011). After washing in PBST overnight, secondary antibodies (Jackson Immuno Laboratories) were applied in PBST for 2–3 days, followed by overnight washes. For cryostat section immunolabeling, embryos were embedded in a solution of 15%/ sucrose and 7.5% gelatin, frozen, and then sectioned at 16 μm using a cryostat (Leica). To melt gelatin off of tissue sections, slides were placed in warm (37–45 °C) 0.1 M phosphate buffer for a couple of minutes. Sections were washed for 30 min to an hour in PBST (0.1% Triton X-100). Primary antibody was applied, and then slides incubated in a humidified chamber for 4 h to overnight. After washing several hours in PBST, secondary antibodies were applied for 2 h, followed by several washes. In situ hybridization for Slit1 was carried out using probes previously described on cryosections (Farmer et al., 2008), followed by Islet1 antibody labeling.

Motor axon tracing with dil

To trace motor axons from mis-positioned motor neurons, embryos were fixed in 4% PFA overnight. Embryos were dissected using fine forceps to remove the skin and mesenchyme, just over the target site. Small crystals were inserted using a fine tungsten needle under a dissecting microscope. The embryos were placed at 37 °C for 1–2 days in 4% PFA to allow the dye to diffuse, and then examined by fluorescence microscopy.

Quantification of motor neuron position defects

The number of Islet-1+ cell bodies in the floor plate was counted under a fluorescence microscope from cryostat sections at the levels of the r1, r4, and brachial spinal cord from Robo and Slit mutant and control embryos on E9.5 and E10.5. The number of total Islet-1+ cells in sections was counted and then the percentage of mis-positioned motor neurons was calculated.

The distance between nIV was used to quantify position of motor nuclei in Netrin-1 or DCC embryos. The measurements were made on Islet-1 labeled whole mount embryos on E10.5. TIFF images of Islet-1 labeled embryos were imported into Image J (Meijering et al., 2004). Varying sizes of embryos were normalized by comparing to the embryo width at the midbrain–hindbrain boundary.