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Kim *et al.*

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Pioneer midbrain longitudinal axons navigate using a balance of Netrin attraction and Slit repulsion

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Abstract

Background: Longitudinal axons grow parallel to the embryonic midline to connect distant regions of the central nervous system. Previous studies suggested that repulsive midline signals guide pioneer longitudinal axons by blocking their entry into the floor plate; however, the role of midline attractants, and whether attractant signals may cooperate with repulsive signals, remains unclear. In this study we investigated the navigation of a set of pioneer longitudinal axons, the medial longitudinal fasciculus, in mouse embryos mutant for the Netrin/Deleted in Colorectal Cancer (DCC) attractants, and for Slit repellents, as well as the responses of explanted longitudinal axons in vitro.

Results: In mutants for Netrin1 chemoattractant or DCC receptor signaling, longitudinal axons shifted away from the ventral midline, suggesting that Netrin1/DCC signals act attractively to pull axons ventrally. Analysis of mutants in the three Slit genes, including Slit1/2/3 triple mutants, suggest that concurrent repulsive Slit/Robo signals push pioneer axons away from the ventral midline. Combinations of mutations between the Netrin and Slit guidance systems provided genetic evidence that the attractive and repulsive signals balance against each other. This balance is demonstrated in vitro using explant culture, finding that the cues can act directly on longitudinal axons. The explants also reveal an unexpected synergy of Netrin1 and Slit2 that promotes outgrowth.

Conclusions: These results support a mechanism in which longitudinal trajectories are positioned by a push-pull balance between opposing Netrin and Slit signals. Our evidence suggests that longitudinal axons respond directly and simultaneously to both attractants and repellents, and that the combined signals constrain axons to grow longitudinally.

Keywords: Axon guidance, Floor plate, Longitudinal axon, Netrin, DCC, Robo, Slit, Hindbrain

Background

Longitudinal axons connect regions of the central nervous system by forming tracts that project long distances by growing precisely along particular trajectories. In the vertebrate embryonic brain stem, longitudinal axons are the first neurons to initiate growth. These axons originate in the midbrain and forebrain, and descend longitudinally through the brainstem to pioneer a simple scaffold of tracts [1-6]. Longitudinal trajectories require a source of cues sustained along their ipsilateral trajectories.

Because they project parallel to the longitudinal axis of the brain stem, a potential source of cues for the pioneer longitudinal tracts is the floor plate tissue along the ventral midline, which produces long range and local cues [7].

Floor plate guidance cues have primarily been studied for commissural axons, which grow toward and across the midline using the floor plate as an intermediate target [8,9]. The major secreted cues for vertebrate commissural axons are Netrin1 via its main attractive receptor Deleted in Colorectal Cancer (DCC) [10-13], and the three Slits via their family of repulsive Robo receptors, primarily Robo1 and 2 [14-16]. Longitudinal axons grow parallel to the floor plate but through the same environment as commissural axons, and could be guided by the same

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attractive and repulsive signals. For repulsion, longitudinal axons respond to the loss of Slit/Robo signals by shifting into the ventral midline [17-20], or show altered fasciculation in other systems [19], and, furthermore, ventral pioneer axons grow around to avoid ectopic patches of Slit-expressing tissue [21,22]. In *Drosophila*, longitudinal axons express different levels or combinations of three Robo isoforms, a Robo code that sets the position of longitudinal axons from the midline [23-25]. A parallel Robo code in mice has been proposed to guide post-crossing spinal cord commissural axons [16,26]. However, Robo1 and 2 appear to guide pioneer longitudinal axons in a redundant, rather than code-like, manner [18,27].

The shift of longitudinal axons into the midline in Slit/Robo mutants implies a strong midline attractive signal. For this attraction, the most likely candidate is Netrin1. Netrin mutations in fly and worm disrupt longitudinal tracts [28-30]. However, Netrin loss in flies causes relatively mild longitudinal errors, and combined mutants suggest that Netrin is not the sole midline attractant accounting for shifts into the midline in Robo mutants [23,31,32]. Other midline attractants for vertebrate pre-crossing commissural axons collaborate with Netrin1 [33,34]. However, the role of midline attractants for longitudinal trajectories remains less defined, as are their potential interactions with Slit/Robo repellents. In zebrafish embryos, Robo2/astray mutations cause shifts of a specific population of dopaminergic longitudinal axons into and across the midline, and this midline attraction is Netrin1-dependent, as it is partially suppressed by morpholino knockdown of either Netrin1 or DCC [20]. However, Netrin1 or DCC knockdowns had no effect in a wild-type background, leaving unresolved whether Netrin/DCC signals have an independent attractive role or are instead only active when Slit/Robo signals are absent [20]. This second mechanism, implying Slit/Robo gating of Netrin1/DCC signaling, is consistent with the predominant silencing model, based on cultured *Xenopus* commissural axons which use a sequential switch from pre-crossing Netrin1/DCC attraction to post-crossing Slit/Robo repulsion [35]. This switch depends on Robo silencing DCC through binding of their intracellular domains [35]. However, in vivo genetic evidence in commissural and other axonal systems show independent action of Netrin and Slit cues [31,36] or even Netrin1 dominance that suppresses or changes Slit responses [37,38], suggesting that Netrin/Slit crosstalk can be diverse.

In this study, we set out to uncover the mechanisms by which longitudinal axons maintain specific trajectories along the dorsal-ventral (DV) axis in embryonic mice. We focused on the medial longitudinal fasciculus (MLF), pioneer axons that are the first to descend through the hindbrain adjacent and parallel to the floor plate. MLF axons are therefore exposed to high levels of Netrin and Slit signals. We tested the roles of Netrin1 and receptor

signaling, and their potential interactions with Slit/Robo signaling, by assaying MLF trajectories in mutant mice, and examining direct in vitro influences on cultured axons. Together, our evidence suggests that Netrin1 attraction and Slit repulsion are balanced against each other to guide and promote the growth of longitudinal pioneer axons along a precise pathway.

Results

Netrin1 is expressed along the path of the medial longitudinal fasciculus

The MLF (medial longitudinal fasciculus) is one of the first tracts to form in the vertebrate brain and becomes a major conduit for fiber populations involved in motor coordination [39]. The MLF is a prototypical longitudinal tract, pioneered by a small group of axons that project in a precise position adjacent and parallel to the floor plate. To evaluate the position of the growing pioneers of the MLF with respect to Netrin1, the Netrin1 expression pattern in the E10 mouse mid- and hindbrain was visualized using in situ hybridization. Netrin1 expression domains include ventral midbrain around the cephalic flexure, intensely in the floor plate of the hindbrain, and a domain of lower expression extending away from the floor plate into the lateral neural tube (Figure 1A). The high floor plate and low lateral expression in the hindbrain closely resembles expression in the spinal cord [10,40].

The MLF pioneers were labeled with neuron-specific β III-tubulin antibody, which showed that the nucleus of the MLF arises in the ventral fore- and midbrain within a region of strong Netrin1 expression. The MLF runs caudally (Figure 1B, arrow) through the midbrain and hindbrain near the Netrin1-positive floor plate. Expression analysis also suggested that the MLF axons were immunoreactive for DCC, the main attractive receptor in many systems [13]. In addition, the hindbrain floor plate expression of Netrin1 overlaps with expression of all three Slit genes [21], which together indicates that the MLF axons are exposed to high levels of both Netrin1 and Slits as they project longitudinally adjacent to the hindbrain floor plate.

Medial longitudinal fasciculus axons deviate dorsally in Netrin1 mutants

To test the role of Netrin1 in the guidance of longitudinal axons, Netrin1^{-/-} mutant mouse embryos were examined during the stage of active MLF outgrowth (embryonic day 10.5, E10.5) using whole-mount labeling with neuron-specific β III-tubulin antibody (Figure 2). Overall, in Netrin1 mutants, the main fascicle of the MLF shifted away from the floor plate compared to wild-type and heterozygous control embryos. Bundles of axons deviated dorsally from the normal ventral position, leading to a wider gap between the bilateral MLF bundles. (Note the

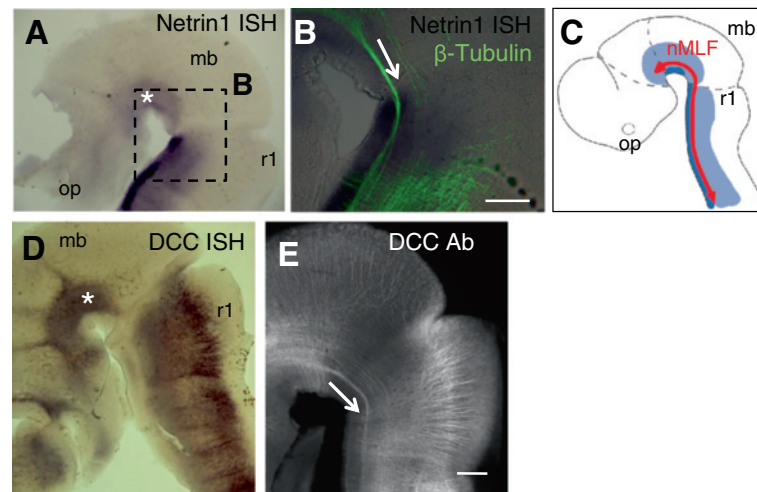


Figure 1 Netrin1 and deleted in colorectal cancer receptor expression is closely associated with the medial longitudinal fasciculus in the early brain. (A-C) Netrin1 expression pattern revealed by in situ hybridization (ISH) in embryonic day 10.5 mouse embryos. Side view of bisected neural tubes. (A) The location of the nucleus of the medial longitudinal fasciculus (nMLF) is indicated (*). (B) Close up showing axons, as labeled by anti- β -tubulin, with respect to Netrin1 expression. The medial longitudinal fasciculus (MLF) travels through the Netrin1-positive region (arrow). (C) Cartoon showing MLF (red) and Netrin1 expression. Dark blue is strong Netrin1 expression while light blue is weaker Netrin1 expression. (D,E) Deleted in colorectal cancer (DCC) expression pattern, revealed by ISH for mRNA (D) and DCC antibody (Ab) labeling (E). The position of the nMLF is noted (*) in (D), and the MLF bundle (arrow) in (E). Scale bar in (B) indicates 200 μ m; scale bar in (E), 200 μ m, applies to (A,D,E). mb, midbrain; op, optic vesicle; r1, rhombomere 1.

MLF errors occurred in conjunction with apparently normal trajectories of hindbrain commissural axons crossing the floor plate in *Netrin1* mutants, suggesting that pre-crossing commissural axons are still attracted toward the ventral midline in the absence of *Netrin1*. We are addressing this issue in a separate study).

The aberrant MLF trajectories in *Netrin1* mutants were also analyzed by diI tracing from the midbrain source of the MLF (Figure 2). In homozygous wild-type or heterozygous controls, the MLF formed a tight bundle of axons against the edge of the floor plate (Figure 2B,D). In *Netrin1* mutants, the MLF axons had dorsal-angling trajectories, and diverged into multiple bundles. The axons in *Netrin1* mutants traveled at a significantly increased distance from the floor plate, almost twice as far away from the midline as normal (Figure 2G; quantification strategy described in Methods, and in Additional file 1). In fact many MLF axons diverged farther than the ventral-most subset included in the quantification. The dorsal shift in the absence of *Netrin1* suggests that *Netrin1* acts as a ventral-ward attractive cue that keeps the MLF near the floor plate. In addition, the wider tract implies that *Netrin1* loss causes a large increase in the variability (wandering) or fasciculation of MLF axons.

The shift in MLF position was also verified by DCC antibody labeling at an earlier stage, E9.5, which showed that the ventral-most DCC+ axons shifted dorsally, and that the overall numbers of longitudinal pioneer neurons was normal in *Netrin1* mutants (Additional file 2). The shift in MLF position was not the result of a wider floor

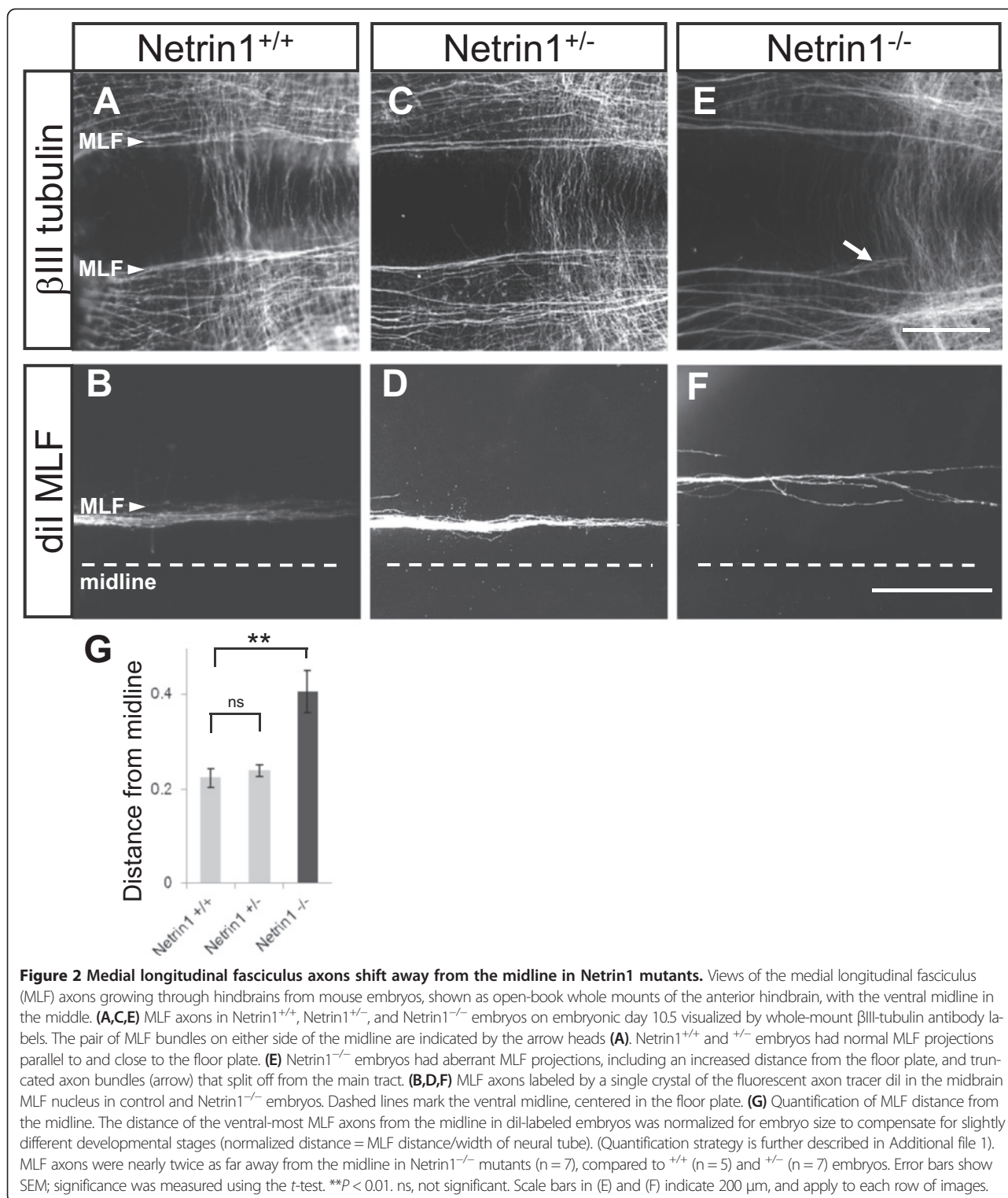
plate, because the floor plate on E9.5 embryos retained the same width and expression of a floor plate marker (Additional file 3).

An additional phenotype in *Netrin1* mutant embryos was the appearance of prematurely truncated bundles of axons (Figure 2E). These bundles were clearly visible in both antibody and diI labels and occurred in both midbrain and hindbrain. All truncated bundles projected at ventral angles. This phenotype was observed in most *Netrin1* mutant embryos but no heterozygous or wild-type embryos. These stalled axons suggest an additional role for *Netrin1* in axon outgrowth promotion.

Medial longitudinal fasciculus axons deviate dorsally in DCC mutants

To test which receptor was mediating *Netrin1* responses in the longitudinal axons, we examined MLF projections in mice with mutations in the main attractive *Netrin* receptor, DCC.

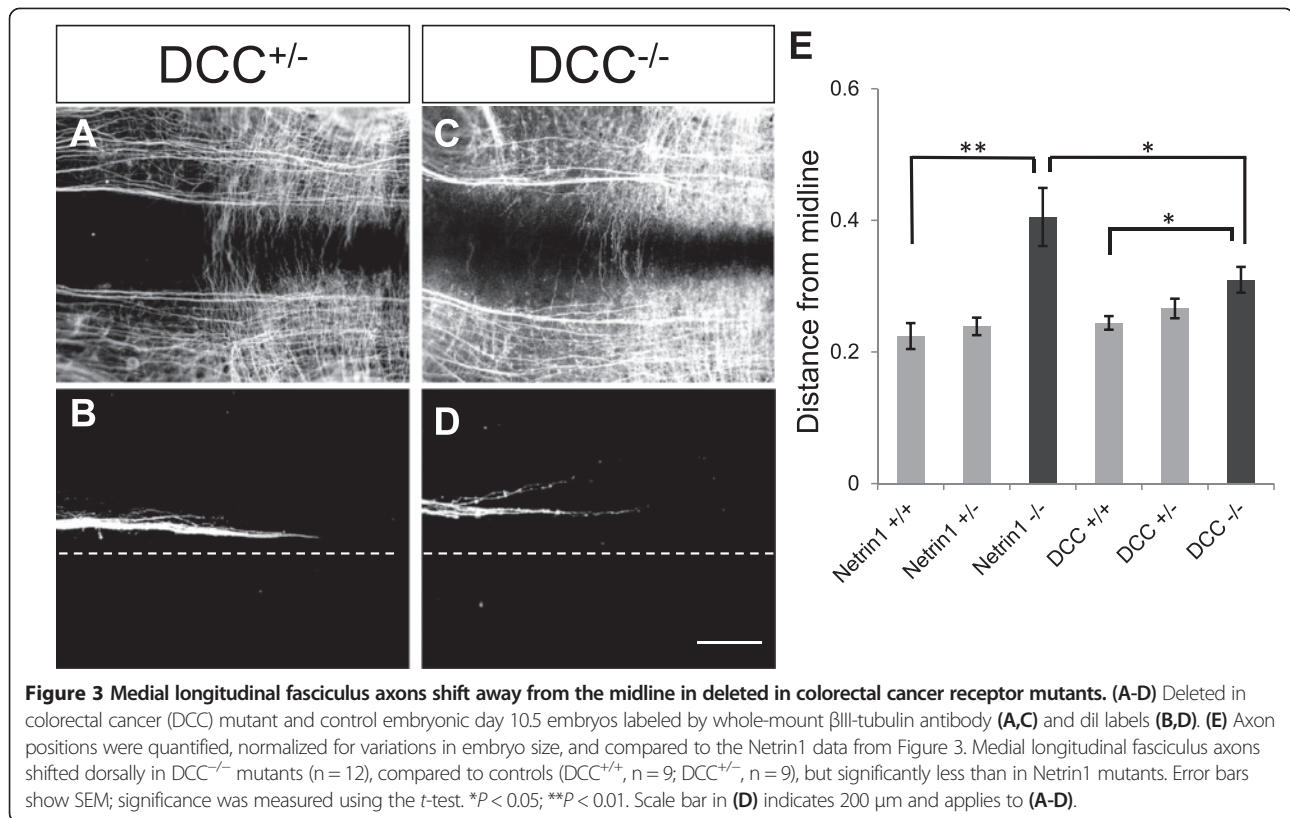
In DCC mutants, MLF axons made errors that were similar to *Netrin1* mutants (Figure 3). MLF axons in DCC mutants shifted away from the floor plate. The average distance was significantly increased over controls, but was also significantly less than the *Netrin1* mutants (Figure 3E). Some bundles deviated dorsally, creating bifurcations in the tract, although the total breadth of the tract appeared more compact than *Netrin1* mutants. The difference between *Netrin1* loss-of-function and DCC loss-of-function suggests that DCC mediates part but not all of the guidance activity of *Netrin1*.



Medial longitudinal fasciculus axons shift toward the floor plate when *Slit* signals are reduced

The shifts of axons away from the ventral midline in *Netrin1* or *DCC* mutants suggest that reduced midline attraction may cause sensitization to midline repellent

signals. *Slit1*, 2, and 3 were good candidate repellents, because our prior study showed that *Slit1* and 2 prevent MLF axons from entering and crossing the midbrain floor plate, at least in the midbrain where these are the only two *Slits* expressed during MLF pioneer navigation



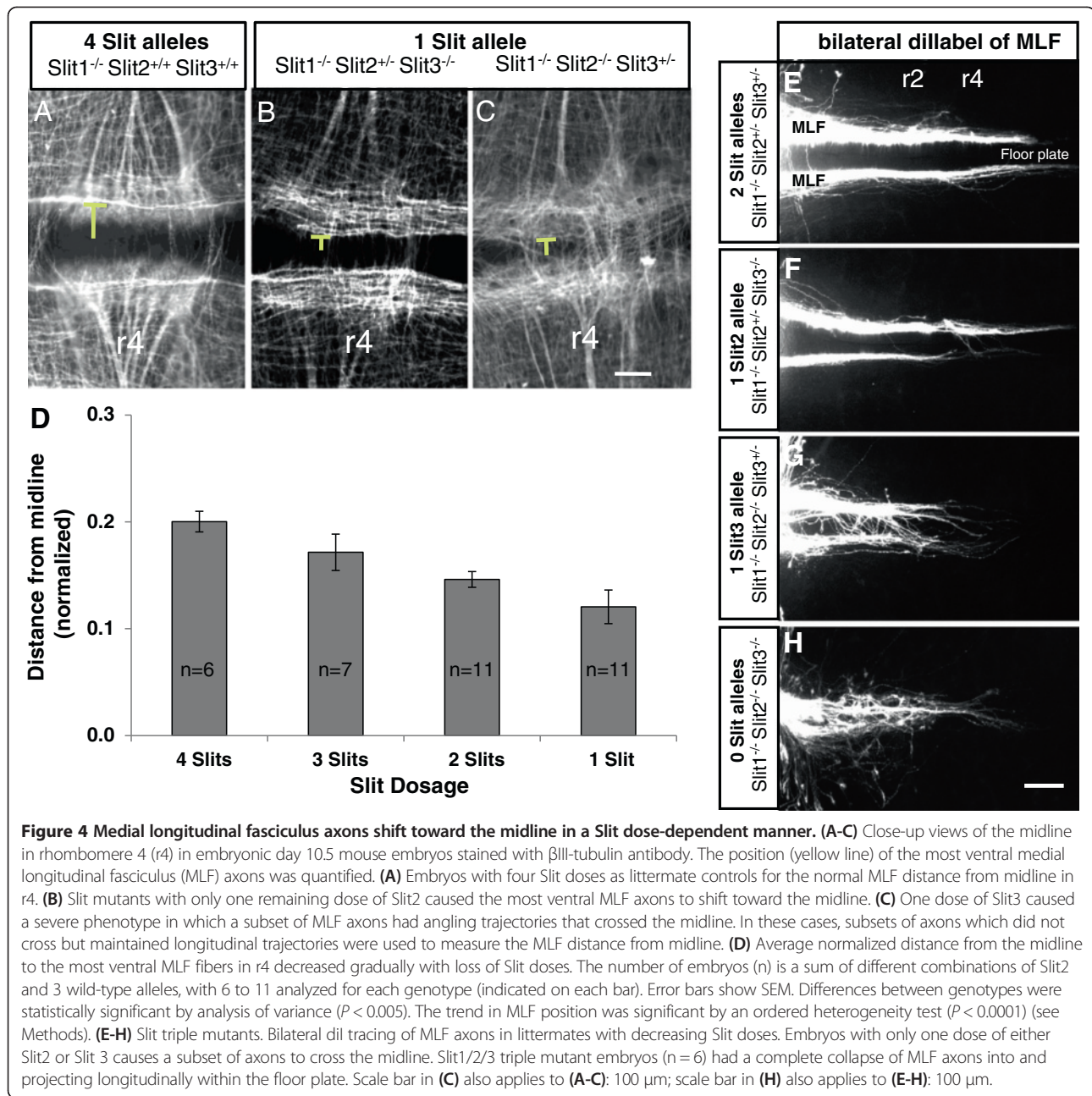
[18]. However, the hindbrain is potentially a different guidance environment, because Slit3 is expressed in combination with Slit1 and 2. Consistent with the potentially overlapping Slit functions in the hindbrain, Slit1/2 double mutants have less severe errors in this region, with some MLF axons crossing but few longitudinal projections within the floor plate itself [18]. These results suggested a midline inhibitory role for Slit1 and 2, but did not give a complete test of Slit function in the hindbrain due to the presence of Slit3. In addition, the previous analysis did not give insights into whether Slits were acting as local non-permissive cues to keep longitudinal axons out of the midline, or whether they acted as diffusible instructive cues to guide ipsilateral trajectories.

Therefore, we undertook an analysis of the genetic function of Slit3, in concert with Slit1 and 2, by analyzing a series of combinations of Slit1, 2, and 3 mutations. Triple homozygous mutants were reported in one prior publication [16].

We reasoned that informative effects on longitudinal trajectories might result from partial reduction of Slit gene dosage, with the prediction that reduced Slit function would lead to a less repulsive floor plate, and cause MLF axons to shift ventrally. To test the effects of reducing Slit expression, we examined mouse embryos with different Slit allele doses (Figure 4). The genetic background of all lines was *Slit1*^{-/-}, which on its own results in normal MLF

projections [18]. By varying Slit 2 and 3 dosage, embryos were produced with one to four functional Slit alleles. Analysis of axon patterns was done primarily with whole-mount β III-tubulin antibody labeling (Figure 4A-D), and axon patterns were further verified using specific dil tracing of the MLF (Figure 4E-H). Ventral shifts in MLF positions were evidenced by narrowing of the space between the flanking MLF bundles, with the strongest effects in rhombomere 4 (r4), where MLF positions were quantified. Reducing Slit dosage resulted in a graded narrowing of the distance between MLF bundles. Embryos with only one functional Slit allele had the largest average ventral shift of the MLF. Interestingly, *Slit1*^{-/-};*2*^{+/-};*3*^{-/-} embryos with a single Slit2 allele had MLF positions farther from the midline than in *Slit1*^{-/-};*2*^{-/-};*3*^{+/-} embryos, which was the most strongly affected genotype. Similar Slit dosage effects were seen in other hindbrain segments, though less dramatically than in r4 (not shown). Tracing of the MLF axons showed that midline crossing occurred in embryos with only 1 or 2 Slit alleles, and again a single Slit2 wild-type allele was more effective than a single Slit3 wild-type allele (Figure 4E,G).

Finally, *Slit1/2/3* triple homozygotes had a strong shift of all MLF axons into the midline, with prominent longitudinal projections within the floor plate (Figure 4H). The shift of MLF axon trajectories into the midline is very similar to the axon errors in *Robo1/2* double



mutants (for example, see Figure 5C), supporting the idea that these two Robo receptors are the primary repulsive Slit receptors for longitudinal axons [18,27]. The graded shift in MLF positions caused by reducing Slit gene dosage implies that the three Slits act together to form a repulsive gradient to push MLF axons away from the midline.

Medial longitudinal fasciculus trajectories in vivo are guided by a balance between Netrin1 and Slit/Robo signals
 The analysis of Netrin/DCC and Slit/Robo guidance of the MLF pioneers suggests that the shifts in the MLF

trajectories in these mutants are caused by an imbalance between attraction and repulsion. In Netrin1 or DCC mutants, the axons shift away from the floor plate, suggesting that repulsion is stronger than attraction. Conversely, the axons shift closer or into the floor plate in Slit or Robo mutants, suggesting an environment in which attraction is stronger than repulsion. However, while the opposing shifts caused by reducing either attraction or repulsion alone are consistent with a balancing mechanism between Netrin and Slit cues, we sought additional genetic evidence for the in vivo relevance of this balance. If the axons shift into the floor plate in Robo1/2 mutants because they are strongly

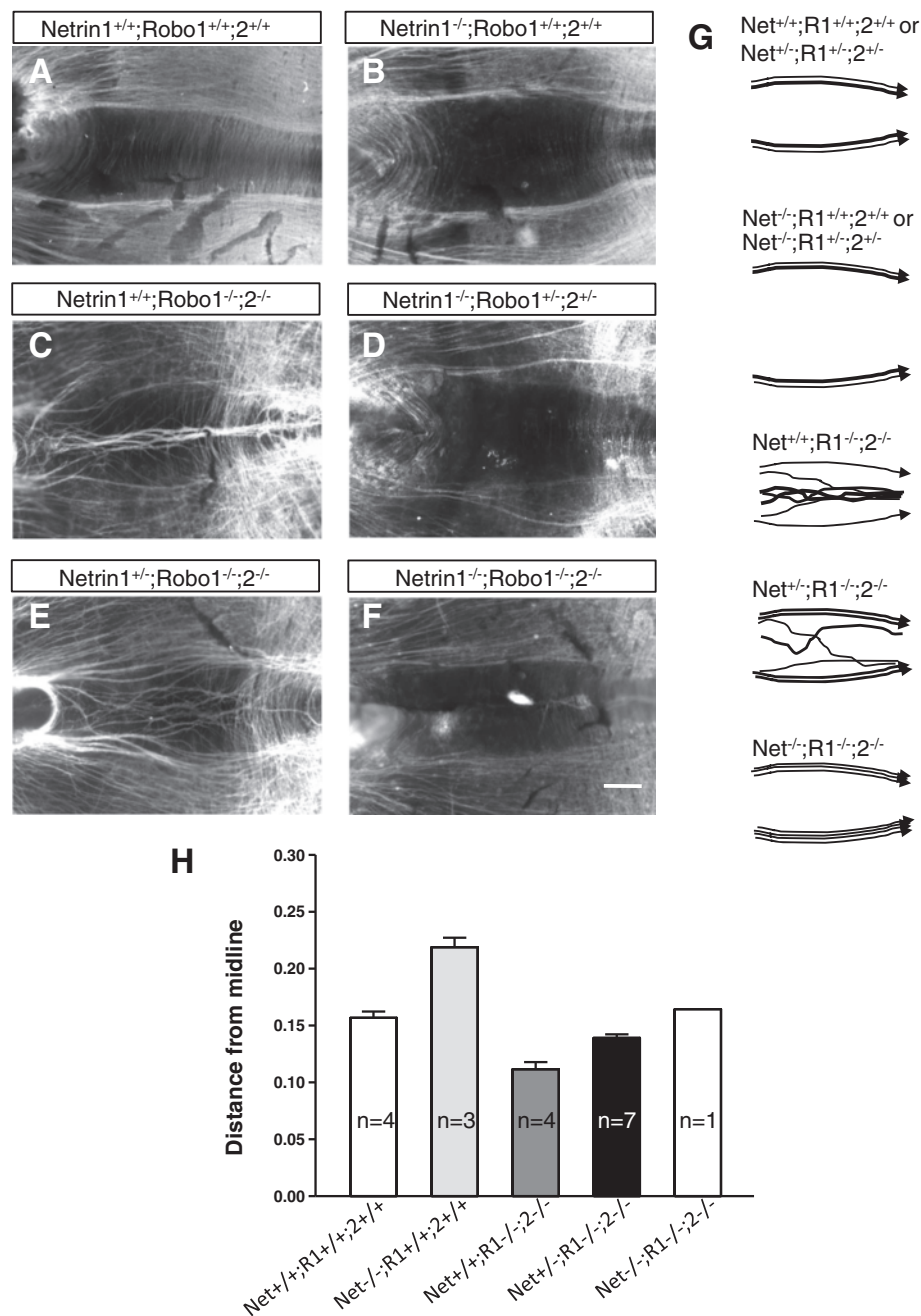


Figure 5 A balance between Netrin and Slit/Robo signaling sets the in vivo position of medial longitudinal fasciculus axons. Combined mutants between Netrin1, Robo1, and Robo2. **(A-F)** Whole-mount open book preparations stained with β III-tubulin antibody. (Key genotypes with significant phenotypes are shown; several classes of heterozygous combinations did not cause medial longitudinal fasciculus (MLF) shifts, and are not shown). **(A-C)** Compared to the control MLF (wild type in **(A)**, $n = 4$), the MLF shifted away from the midline in Netrin1^{-/-} mutants (**(B)**, $n = 3$). In Robo1/2 mutants, the MLF shifted toward the midline and, in addition, many axons entered and grew longitudinally within the midline (**(C)**, $n = 4$). **(D)** When Robo1/2 function was reduced in a Netrin1 mutant background, Netrin1^{-/-}; Robo1^{+/+};2^{+/+}, the MLF maintained a similar dorsal shift ($n = 1$). **(E)** When Netrin1 function was reduced in a Robo1/2 mutant background, Netrin1^{+/+}; Robo1^{-/-};2^{-/-}, the MLF tracts shifted to a near-normal position, and the number of midline axon bundles was reduced ($n = 7$). **(F)** A single triple mutant embryo was isolated, showing an MLF pattern similar to wild-type, with neither dorsal or ventral shifts ($n = 1$). **(G)** Schematic summary of longitudinal pioneer patterns in key genotypes. **(H)** Graph of average normalized distance from the midline to the most ventral MLF fibers. All means differed significantly by analysis of variance ($P < 10^{-7}$), and by pair-wise t -tests. Scale bar in **(F)** also applies to all: 100 μ m.

attracted to Netrin1, then a reduction in Netrin1 attraction is predicted to suppress the shift of axons into the floor plate. Similarly, the shift of axons away from the floor plate in Netrin1 mutants should be alleviated by a reduction of Slit/Robo repulsion.

We took an *in vivo* genetic approach by attempting to make combined mutants between the two guidance systems, which have not previously been reported. Practical considerations guided us to make Netrin1; Robo1;2 triple mutants. We chose Netrin1 mutants to test attraction, because the homozygous Netrin1 mutant phenotype was stronger than DCC. For testing Slit/Robo repulsion, Robo1/2 mutants were used because the two genes are closely linked and segregate as one mutant allele, simplifying the genetic crosses. Importantly, genetic evidence suggests that Robo1 and 2 are the dominant Slit receptors for MLF axons, because the axons strongly enter the floor plate in Robo1/2 homozygous mutants [18,27], which is similar to Slit1/2/3 triple mutants (Figure 4).

When Netrin1 function was reduced in the Robo1/2 mutants (that is, Netrin1^{+/-}; Robo1^{-/-};2^{-/-}) both of the Robo1/2-dependent MLF axon phenotypes were less severe (Figure 5E). The remnant MLF shifted back nearly to the wild-type position, with larger, more prominent bundles. In addition, the longitudinal bundles within the midline were less prominent and involved fewer axons. This suggests that the MLF midline entry and ventral shift in Robo1/2 mutants is dependent on ventral-ward Netrin1 attraction.

We also attempted to reduce Robo1/2 function in Netrin1 homozygous mutants. Unfortunately, after many litters from crosses between triple heterozygotes producing a total of 99 embryos, the distribution of genotypes indicated a strong lethal genetic interaction between the Netrin1 and Robo1/2 mutant alleles. In the Netrin1^{-/-} background, when Robo1/2 gene dosage was reduced by half (that is, Netrin1^{-/-}; Robo1^{+/-};2^{+/-}) only one embryo was isolated (1/99), instead of a predicted 1/8 ratio. The low yield suggests a genetic requirement of all three genes for viability at an embryonic stage prior to E10.5. We also set up lines to combine Netrin mutations with either single Robo mutant (that is, Netrin1^{-/-}; Robo1^{+/-} and Netrin1^{-/-}; Robo2^{+/-}) but these also produced lethal interactions because we were unable to isolate double homozygotes from either cross (not shown). We speculate that the Netrin/Robo lethal interaction may involve non-neuronal tissues, such as tissue movements during gastrulation [41,42], or early vascular or placental development [43-46]. Nonetheless, in the unique Netrin1^{-/-}; Robo1^{+/-}; 2^{+/-} mutant embryo, the MLF retained a shift away from the midline similar to Netrin1^{-/-} mutants (Figure 5D). This result suggests that Robo1/2 heterozygotes retain sufficient Slit/Robo repulsion to push the MLF axons away from the midline.

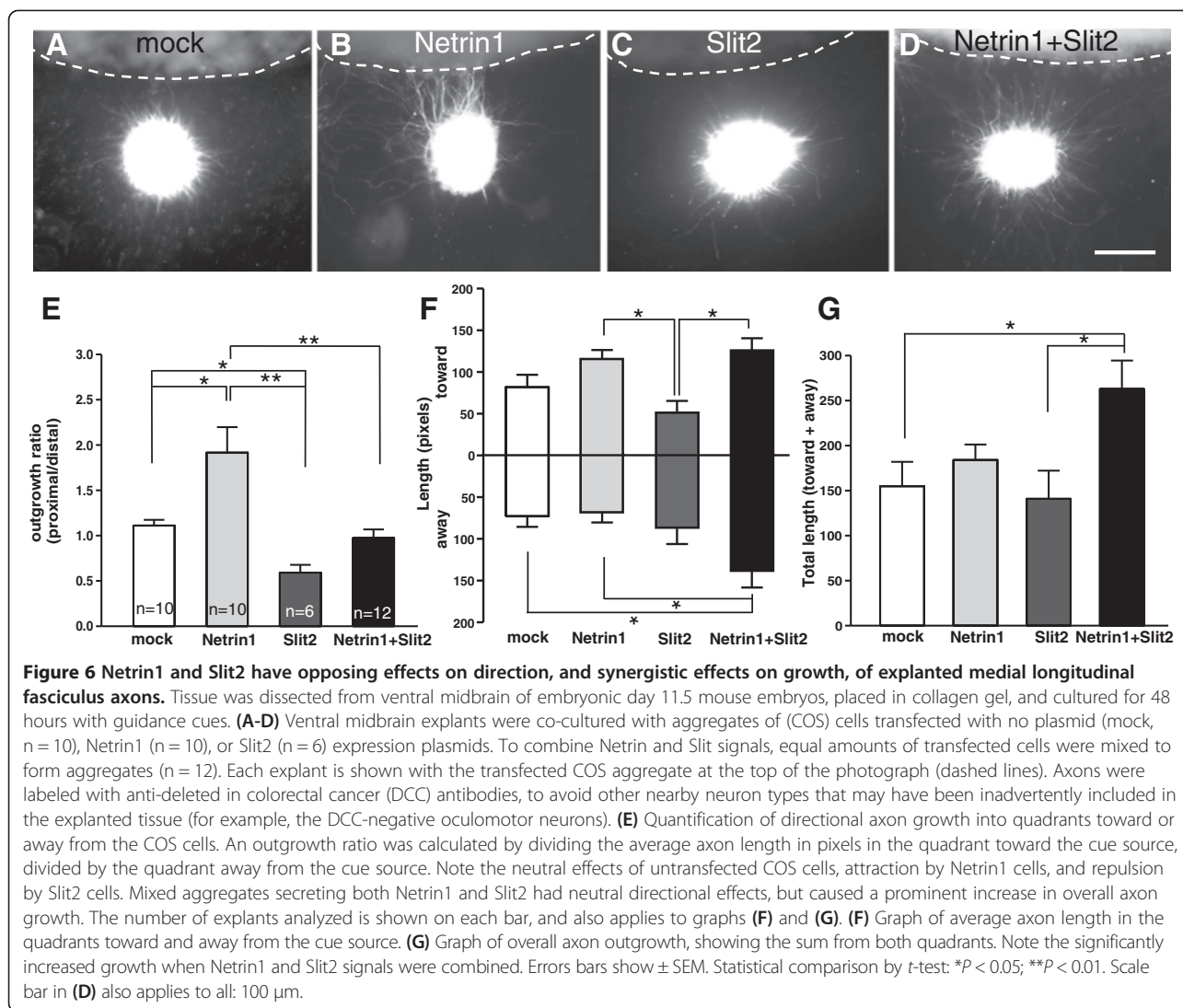
The ultimate goal of combining Netrin1, Robo1, and Robo2 mutations also unfortunately resulted in only a single triple homozygote, Netrin1^{-/-}; Robo1^{-/-};2^{-/-}, identified on E10.5 (thus also 1/99 instead of a predicted 1/16). Strikingly, this remaining embryo had MLF tracts formed at positions close to wild-type (Figure 5F). No MLF axons entered or projected longitudinally within the floor plate. Thus, the Netrin1 mutant shift away from the midline was suppressed by removing Robo1/2 repulsive function. A complementary interpretation is that the Robo1/2 mutant shift toward and into the midline was suppressed by removing Netrin1 attractive function. The axon patterns are consistent with the partial restoration of MLF position in Netrin1^{+/-}; Robo1^{-/-};2^{-/-} mutants. Together, these observations of combined Netrin1 and Robo1/2 mutants are consistent with a guidance mechanism in which Netrin1 and Slit/Robo signals act in balance to position the MLF axon trajectories.

As a side note, to attempt to bypass the triple mutant lethality, we also combined Netrin1 mutations with each individual Robo (that is, Netrin1^{+/-}; Robo1^{+/-} crosses in a Robo2^{+/-} genetic background, and Netrin1^{+/-}; Robo2^{+/-} crosses in a Robo1^{+/-} genetic background). Although we did not pursue this as exhaustively as for the triple mutants, no double homozygous mutants in either background were identified (not shown), suggesting the same lethal genetic interaction between Netrin1 and either Robo1 or Robo2.

Medial longitudinal fasciculus axons can react directly in culture to combined Netrin1 and Slit signals

The analysis of mutant mice indicates that Netrin and Slit signals have important genetic functions in positioning MLF axons *in vivo*. These results specifically imply that these longitudinal axons can respond to both attractive and repulsive signals simultaneously. However, the *in vivo* phenotypes could result from direct or indirect effects. To test for direct effects of isolated Netrin1 and Slit signals, we established an explant culture system for MLF axons. Small pieces of ventral midbrain tissue containing the nucleus of the MLF were embedded in collagen gels and cultured. Although specific molecular markers for MLF axons are not available, all axons growing out of the explants did label with DCC antibodies as expected for the MLF (Figure 1D; Figure 6).

Ventral midbrain explants were co-cultured in the presence of COS cells transfected with secreted forms of Netrin1 or Slit2 (Figure 6). Axons cultured in the presence of control COS cell aggregates transfected with YFP had no directional influence. Netrin1-transfected COS cells had a positive effect on the direction of axon growth: axons grew toward Netrin1-expressing aggregates. In contrast, MLF axons had strong inhibitory responses to Slit2 because they grew away from Slit2-expressing



aggregates. These effects suggest that MLF axons can respond directly to Netrin and Slit signals, with opposing responses consistent with their *in vivo* effects.

To more closely reflect the situation *in vivo*, explants were cultured adjacent to COS aggregates secreting both Netrin1 and Slit2 (Figure 6). The presence of Netrin1 counteracted the repulsive effects of Slit2, resulting in symmetrical growth, which is evidence that MLF axons can simultaneously react to the two opposing signals. Surprisingly, the axons of explants exposed to an overlapping gradient of both Netrin1 and Slit2 were longer than those challenged with either single cue (Figure 6G). This indicates a synergistic effect of Slits and Netrin1 on MLF outgrowth, in addition to the individual instructive positive and negative signals. Together, the explant experiments indicate that Slits and Netrin1 cooperate to both guide and promote the outgrowth of MLF axons.

Discussion

Longitudinal axon trajectories maintain precise positions relative to the DV axis of the neural tube, so rather than having one decision point at an intermediate target, longitudinal axons need continuous guidance input. Our results suggest that pioneer MLF trajectories are set by the combined action of Netrin1 and Slit signals. This navigation mechanism depends on a counter balance of attractive and repulsive cues, integrated simultaneously in a “push-pull” manner, to guide axons longitudinally at a specific position. MLF axon responses in culture further suggest that the combined signals synergistically increase the growth rate of these axons. The importance of a balance between these opposing cues is supported by several lines of evidence.

Our first major finding was that Netrin1 loss caused longitudinal axons to wander dorsally. Even the ventral-most axons in mutant embryos were significantly farther away from the floor plate than wild-type axons, and many

axon trajectories diverged farther. Additionally, bundles with prematurely truncated axons indicate a growth-promoting function, which was further supported by observation of outgrowth towards a Netrin1 source *in vitro*. Netrin1 has at least two positive effects on MLF axons, which together serve to position and promote the growth of the MLF near the floor plate. A major Netrin receptor for longitudinal axons appears to be DCC, because DCC mutant axons shifted significantly away from the midline.

Slit signaling is also critically important for positioning longitudinal pioneer axons as an opposing repellent. Our previous study of Slit1/2 mutant mouse embryos showed that Slits are required to prevent MLF entry into and crossing of the floor plate in the midbrain [18]. Blocking midline entry could be accounted for by a local inhibitory role of Slits within floor plate tissue. Here, we extended these findings to show that reduced Slit gene dosage causes MLF axons to shift toward the floor plate. This observation implies that Slits can act not just locally but at a distance, and in a graded manner. As Slits can also act at a distance on axons in culture, together these results suggest that Slit signals position MLF axons by repelling them away from the ventral midline.

Since MLF axons respond to the Slits and Netrin1 simultaneously along their trajectory, we propose that a combination of repulsive and attractive cues position pioneer MLF axons to navigate within a narrow zone adjacent to the floor plate. MLF axons are pushed away from the floor plate by Slit signals, and pulled toward the floor plate by Netrin1 signals. This model explains why weakening either the positive or negative signals causes MLF shifts: releasing axons from the ventral attraction causes dorsal shifts driven by Slit repulsion, and releasing axons from the ventral repulsion causes ventral shifts driven by Netrin attraction. To formally test this mechanism, we combined mutations between Netrin1 and Robo1/2, which partially or wholly suppressed the axon errors caused by loss of either of the single pathways. The positive synergy of Slit and Netrin on outgrowth rates *in vitro* further indicates that MLF axons can respond simultaneously to both sets of cues, and consistent with the region near the floor plate providing a growth-promoting environment for the rapidly advancing MLF axons. Another important implication is that silencing via a Robo-DCC hierarchy does not appear to occur in longitudinal axons. The key prediction of the silencing model is that if Slit/Robo signaling silences DCC-mediated attraction, then the presence or absence of DCC (or Netrin1) should have no effect on MLF guidance. However, mutants for either Netrin1 or DCC have major MLF shifts, showing that Netrin1/DCC signaling is active and critical for MLF navigation, despite the fact that they are growing next to the floor plate where they are exposed to the highest possible levels of Slit repulsion.

The mechanism of longitudinal guidance by balanced Netrin and Slit cues in mouse embryos extends, in several ways, a previous report on the guidance of longitudinal axons through the zebrafish hindbrain [20]. That study showed that a different longitudinal axon population, which is dopaminergic and descends through the hindbrain at a more dorsal position, shifted toward the ventral midline in Robo2/astray mutants [20], consistent with Robo guidance being critical to keep mouse longitudinal axons out of the midline [18]. However, single knockdowns of either zebrafish Netrin, DCC, or combinations of the Slits did not perturb longitudinal guidance in a wild type genetic background [20]. The zebrafish experiments did suggest that Netrin/DCC and Slit/Robo signaling was involved in the Robo2-dependent positioning of tracts, because Netrin or DCC morpholino knockdown in Robo2 mutants was effective at suppressing the mutant shift toward the midline. Our results provide a more comprehensive test of how these cues guide the mouse MLF, because mutants for Netrin1, DCC, or the Slits do have longitudinal shifts, and explants also confirmed that the cues can act directly on cultured axons. The absence of zebrafish phenotypes for several of the key molecules may be due to partial knockdown via morpholinos, which agrees with our observations that heterozygous mouse embryos maintained normal tract positions. However, it should also be noted that the zebrafish mutants or knockdowns perturbed only a specific dopaminergic axon subpopulation, while strikingly the zebrafish MLF axons appeared to project normally [20]. The comparison of these mouse and zebrafish studies emphasizes that a good understanding of longitudinal guidance mechanisms will require additional axon populations to be studied in a variety of experimental approaches.

An interesting implication of the push-pull mechanism is that it provides a general strategy for DV positioning of axon populations, where increased relative attraction to Netrin1 could override Slit repulsion to result in a ventral position, or increased repulsive responses to Slits would result in more dorsal positions. For example, increases in Robo3 expression appear to interfere with Robo-mediated repulsion to cause shifts in zebrafish longitudinal tract positions [47]. However, evidence suggests that shifts in axon responses are unlikely to be simply regulated by receptor levels on axons or growth cones. On one hand, MLF axons show the brightest DCC labeling of the pioneer descending tracts, which could mediate strong attraction ventrally. On the other hand, Robo1 expression also appears highest in MLF [18,27], so the position of the MLF as the ventral-most tract is not simply explained by an imbalance of attractive receptors over repulsive receptors. This suggests that receptor signaling may be modulated in other ways such as cell surface localization or downstream signaling modulators.

Longitudinal navigation involves the integration of positive and negative cues that are opposed, and potentially confusing. Binding of either Slits or Netrin to their receptors sets off a cascade of intercellular events that affect multiple pathways [48,49]. Interestingly, Netrin and Slit signal transduction pathways have opposing effects on common components such as the small GTPase Cdc42 [50,51]. Other potential switches between attraction and repulsion include cyclic nucleotide levels and calcium levels [52]. Thus, converging signals could be integrated to provide a fine-tuned response to opposing Slit and Netrin signals. Future experiments on receptor signaling mechanisms and the cell biology of growth cones will be needed to reveal integration mechanisms for longitudinal guidance.

The restoration of a normal MLF projection pattern in the combined Netrin1/Robo1/2 triple mutant is an intriguing example of the ability of axons to continue to navigate, even in a “ground-state”, devoid of Netrin1/Slit guidance. That the axons can overcome the loss of their main guidance cues suggests that additional signals likely also play roles in longitudinal navigation. These may include other midline attractants such as Sonic hedgehog (Shh) or vascular endothelial growth factor (VEGF) [33,34], midline repellents such as Semaphorins [53], and directional cues along the anterior-posterior axis such as Wnts or Shh [54-57]. Axon-axon interactions also play a role in regulating the cohesion of longitudinal tracts [19]. Further evidence that long range cues can act by modulating axonal expression of adhesion molecules suggest important interactions between long range and local guidance mechanisms [53].

Studies from a range of systems suggest that Netrin and Slit cues can be interpreted by axons in diverse ways. In fact, the initial nematode Netrin mutants were identified by errors in circumferential axons [28], which grow away from Slit and toward Netrin, implying both cues act simultaneously and cooperatively on these axons [29,58,59]. In *Drosophila*, Slit and Netrin cues are secreted together from the midline, but genetic analysis suggests that they signal in parallel in an additive manner, rather than in a hierarchical silencing mechanism [31]. Other systems have hierarchical cue interactions which are quite distinct from silencing. For example, mouse thalamocortical axons are repelled when presented by Slits alone, and do not react to Netrin1 alone, but Slit switches to attraction in the presence of Netrin1 to promote projections into the rostral cortex [37]. This switch in thalamocortical responses requires a novel Robo1 co-receptor, FLRT3, which acts to upregulate DCC as an attractive Netrin1 receptor [60], but it remains unknown whether these receptor interactions have wider roles in other axon populations. Netrin and Slit cues interact differently in the mouse corpus callosum, where

neocortical axons again do not react to Netrin1 alone, but Netrin1/DCC signaling instead attenuates Slit repulsion, allowing midline crossing [38]. Similarly, DCC inhibits Slit/Robo repulsion of precrossing axons in zebrafish spinal cord, allowing them to approach the midline [61]. Together, the diversity of potential axonal reactions to just this pair of cues is impressive, as their interactions can be hierarchical in either direction, cause switching of repulsion to attraction, or can be balanced in a non-hierarchical fashion, as we show for longitudinal pioneers. It seems likely that additional novel interactions between axon guidance signals are critical for axon circuit formation.

Conclusions

Our evidence indicates that longitudinal axons navigate using simultaneous Netrin1/DCC and Slit/Robo signals to set their trajectories. This push-pull mechanism suggests that longitudinal axons integrate the opposing signals through a balance that constrains their growth into longitudinal trajectories at specific positions. Overall, our results build upon the model that the molecular landscape of guidance cues laid out in embryonic brain tissue is interpreted in various ways by different types of axons to generate diverse and specific patterns of tracts to wire the brain.

Methods

In situ hybridization

Whole mount in situ hybridization was carried out using standard procedures [62]. Probes for Netrin1 and Slit1 were provided by Marc Tessier-Lavigne (Rockefeller University, New York, NY). DCC probes were generated by reverse transcription-PCR, using procedures previously described [63].

Immunohistochemistry

Neural tubes were dissected from the rest of the embryo, and washed for several hours in PBS containing 10% FBS and 1% TritonX (PBT + serum). Primary antibody in PBT + serum was applied for 1 to 3 days: rabbit anti- β III tubulin (Covance, Princeton, New Jersey; 1:1000), goat anti-DCC (Santa Cruz, Dallas, TX; 1:250). After washing in PBT + serum overnight, secondary antibodies (Jackson ImmunoLabs, West Grove, PA) were applied in PBT + serum at 1:200 for 1 to 2 days, followed by overnight washes.

Mouse embryos

Animal experiment protocol #00435 was approved by the University of Nevada, Reno, IACUC, following guidelines of the National Institutes of Health, and accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. Expression analyses were performed with CD1 embryos. Netrin mutants were previously described, and genotyped by a combination of PCR amplification of the lacZ gene trap insert,

intensity of Xgal labeling of embryonic spinal cord tissue (see Additional file 4), or examination of commissure formation in the embryonic spinal cord [64]. The *Netrin1*^{-/-} hindbrain floor plate appeared normal using the 4C7 antibody (DSHB) against HNF3b/FoxA2. DCC mutants were previously described, and genotyped by PCR [13]. *Slit1*^{-/-}; *Slit2*^{+/-}; *Slit3*^{+/-} triple mutant founder mice were a gift from Marc Tessier-Lavigne, (Rockefeller University) [15,16,65], and were mated to produce embryos with *Slit1*^{-/-} genotypes combined with various doses of *Slit2* and *Slit3*.

To obtain *Netrin1/Robo1/Robo2* triple mutants, *Netrin1*^{+/-} heterozygotes were first mated with mice carrying the linked *Robo1/2* heterozygous mutations, and then the resulting *Netrin1*^{+/-}; *Robo1*^{+/-}; *Robo2*^{+/-} triple heterozygotes were intercrossed to maintain the new strain and to generate embryos. The embryos were genotyped by PCR of both *Robo1* and 2 alleles, combined with histological examination of commissure formation in the embryonic spinal cord to verify the *Netrin1* genotype. It is unlikely that the rarity of the triple mutant combination (1/99) was caused by a general vascular defect, because the embryos did not appear hypoxic within our triple het litters of embryos, and no “moles” were included in our analysis. Second, somites were counted for all of the embryos analyzed, and were within the expected range of development.

Embryos were collected on E10 or E10.5, with noon of the day of the vaginal plug designated as E0.5. The lipophilic fluorescent axon tracer DiI was used as previously described [5,66] to label the midbrain nucleus of the MLF and trace MLF axon trajectories as they descended through the hindbrain.

Quantification of normalized distance from the midline

The distance of the ventral-most axons from the midline in DiI and β III-tubulin labels was measured at regular intervals along the length of the neural tube using ImageJ from NIH (Bethesda, MD; see Additional file 1). For the Netrin analysis, to normalize for embryo size, distance from the midline was divided by the total DV width of the neural tube as measured at the boundary between r1 and r2. The average normalized distance for each embryo was then used to perform statistical analysis by analysis of variance (ANOVA), which found that the means were significantly different, and by *t*-test.

For the analysis of MLF position in *Slit* mutants, measurements between the MLF fascicles of β III-tubulin labeled embryos were halved to represent average distance from the midline, and expressed as ratios as above. The numbers were pooled for *Slit* wild-type allele dose, ranging from 4 to 1. The four groups of means were compared by ANOVA, which found that the means were significantly different at $P < 0.005$. As another statistical

comparison, the decreasing trend of the means as *Slit* doses decrease from 4 to 1 was analyzed by an ordered heterogeneity test [67], in which the expected rank order was found to be the same as the observed rank order, with a confidence level of $P < 0.0001$.

The MLF position in *Netrin1/Robo1/Robo2* triple mutants was quantified by the same strategy, and the means for different genotypes were found to be significantly different by ANOVA ($P < 10^{-6}$), and by appropriate pairs of *t*-tests.

Explant co-culture

Ventral mid- and forebrain tissue of E11.5 embryos was dissected to include the source of the MLF, and to exclude the medial-most tissue containing motor neurons of nIII. Culture conditions for explants were as described previously [33]. To provide localized *Netrin1* or *Slit* sources, COS cells were transfected with a chick *Netrin1* expression plasmid (gift of Marc Tessier-Lavigne, Rockefeller) [11] or a human *Slit2* expression plasmid (gift of Yi Rao, Peking (Beijing, China)) [68]. COS cells were cultured for 24 hours, then aggregates formed and placed near tissue explants. Combined *Netrin1* and *Slit2* signals were provided by mixing equal amounts of cells transfected with each plasmid. Expression of the cues was verified using anti-myc antibodies to label fixed COS cell cultures. Explants were cultured for 48 hours, and then fixed. Anti-DCC or β III-tubulin antibody was used to visualize MLF axons. The length of axons was measured using the ImageJ plugin NeuronJ to determine mean axon lengths from each explant, with the number of axons ranging from 7 to 62. For directional analysis, quadrants were marked on images using Adobe Illustrator (San Jose, CA), then imported and quantitated in Image J.

Additional files

Additional file 1: Quantification of MLF distance from the midline.

Example showing the measurements made to compare the distance of MLF axons from the midline. Differences in developmental stage were normalized relative to the width (large bracket, wr1r2) of neural tissue at the r1/r2 border.

Additional file 2: Netrin1 mutant longitudinal axons retain wild-type axon numbers and DCC expression.

(A-C) Whole mount embryos on E9.5 labeled with DCC antibody. The DCC labeling intensity was consistently lower in *Netrin1*^{+/-} control embryos. (D) Quantification of numbers of longitudinal axons on E9.5, by counting longitudinal axons on each side of the hindbrain in images of whole mounts. The n numbers indicate the number of embryos analyzed for each genotype; the error bars indicate SEM. By ANOVA analysis, there were no significant differences in axon number between the genotypes. Both ventral and dorsal axons were included in these counts. The size of the most ventral bundle, consisting of the MLF axons, was similar between genotypes, but tight fascicles made it difficult to determine the number of individual axons. Scale bar: 100 μ m.

Additional file 3: Netrin1 mutants retain normal hindbrain floor plate size and specification.

Sections through E9.5 hindbrain labeled with the 4C7 antibody against HNF3b/FoxA2, a transcription factor expressed in a domain including the floor plate and adjacent ventral

cells. The morphology and size of the floor plate domain appears similar in controls and Netrin1 mutants. Scale bar: 100 μ m.

Additional file 4: Genotyping of Netrin1 mutant embryos.

Beta-galactosidase antibody labeling of spinal cord sections. Note the higher intensity of antibody labeling in homozygous mutants. Scale bar: 100 μ m.

Abbreviations

ANOVA: analysis of variance; COS: CV-1 (simian) in Origin, and carrying the SV40 genetic material; DCC: deleted in colorectal cancer; DV: dorsal-ventral; E: embryonic day; FBS: fetal bovine serum; MLF: medial longitudinal fasciculus; PBS: phosphate-buffered saline; PBT: PBS containing 1% Triton X; PCR: polymerase chain reaction; r4: rhombomere 4; Shh: Sonic hedgehog; VEGF: Vascular endothelial growth factor.

Competing interests

The authors declare that they have no competing interests.

Author' contributions

MK and WTF contributed equally to the manuscript, and are listed as co-first authors. MK, WTF, BB, and SAM contributed to the conception/design of the study, the acquisition, analysis, and interpretation of data, and edited the manuscript. PJF contributed to the acquisition of data and edited the manuscript. FC contributed to the conception/design of the study, interpretation of data, and edited the manuscript. GSM contributed to the conception/design of the study, the acquisition, analysis, and interpretation of data, and edited the manuscript. All authors read and approved the final manuscript.

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