Robo–Slit interactions regulate longitudinal axon pathfinding in the embryonic vertebrate brain

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Received for publication 20 August 2007; revised 1 October 2007; accepted 22 October 2007
Available online 7 November 2007

Abstract

The early network of axons in the embryonic brain provides connectivity between functionally distinct regions of the nervous system. While many of the molecular interactions driving commissural pathway formation have been deciphered, the mechanisms underlying the development of longitudinal tracts remain unclear. We have identified here a role for the Roundabout (Robo) family of axon guidance receptors in the positioning of longitudinally projecting axons along the dorsoventral axis in the embryonic zebrafish forebrain. Using a loss-of-function approach, we established that Robo family members exhibit complementary functions in the tract of the postoptic commissure (TPOC), the major longitudinal tract in the forebrain. Robo2 acted initially to split the TPOC into discrete fascicles upon entering a broad domain of Slit1a expression in the ventrocaudal diencephalon. In contrast, Robo1 and Robo3 restricted the extent of defasciculation of the TPOC. In this way, the complementary roles of Robo family members balance levels of fasciculation and defasciculation along this trajectory. These results demonstrate a key role for Robo–Slit signaling in vertebrate longitudinal axon guidance and highlight the importance of context-specific guidance cues during navigation within complex pathways.

Keywords: Roundabout; Slit; Axon guidance; Forebrain; Zebrafish; Morpholino

Introduction

Neuronal growth cones follow specific pathways and navigate a series of choice points to establish appropriate connections. At choice points, growth cones interpret a combination of short- and long-range cues, which are perceived as either attractive or repulsive (reviewed in Tessier-Lavigne and Goodman, 1996). The net response of the growth cone to these guidance cues depends not only on the complement of receptors present, but more importantly, on the way in which these receptors regulate the activity of one another in a context-dependent manner. In Xenopus spinal cord neurons, binding of Slit2 to Robo1 initiates the formation of a receptor complex with DCC. This interaction is sufficient to silence chemotraction mediated by Netrin-1 (Stein and Tessier-Lavigne, 2001). More recently, Robo3/Rig-1 has been shown to antagonize Robo responsiveness to the chemorepellent Slit in pre-crossing commissural axons, allowing them to enter the floor plate and cross to the contralateral side (Sabatier et al., 2004). An understanding of such interactions is providing insight into how growth cones interpret multiple and potentially conflicting cues in order to navigate complex pathways.

The Roundabout (Robo) transmembrane proteins constitute a phylogenetically conserved family of axon guidance receptors, which together with their repellant ligand Slit, have been demonstrated to perform important roles in the regulation of axon guidance decisions (Battye et al., 1999; Brose et al., 1999; Kidd et al., 1999; Rajagopalan et al., 2000b; Simpson et al., 2000a). Three Robo receptors (Robo1, Robo2 and Robo3/Rig-1) and three Slit ligands (Slit1, 2 and 3) are expressed in the vertebrate nervous system (Itoh et al., 1998; Kidd et al., 1998; Yuan et al., 1999). The three vertebrate Slit homologs appear to signal redundantly to control commissure formation in the spinal cord since only in Slit1, 2 and 3 triple mutant animals are commissural guidance defects revealed (Long et al., 2004).
Additionally, as members of the IgCAM superfamily, Robo receptors have also been shown to be capable of both homophilic and heterophilic interaction through their extra-cellular domains (Hivert et al., 2002). In vitro analyses suggest that this Slit-independent interaction is sufficient to increase axon outgrowth and fasciculation (Hivert et al., 2002). The extent to which these adhesive interactions may contribute to the function of Robo family members to guide axons in vivo has not yet been directly tested.

Evidence from the ventral midline of Drosophila and the vertebrate spinal cord supports an evolutionary conserved role for Robo–Slit signaling to regulate commissural axon guidance (Kidd et al., 1999; Rajagopalan et al., 2000a; Sabatier et al., 2004; Simpson et al., 2000b). Robo expression is initially kept low (or masked by Robo3/Rig-1 in higher vertebrates) on contralaterally projecting axons as they approach the midline, and later upregulated (or unmasked) to prevent lingering and subsequent recrossing. By contrast, ipsilaterally projecting axons express high levels of the Robo receptor and consequently are unable to cross the midline. In this way, Robo not only acts in a sorting capacity to specify which axons cross the midline but also ensures that axons continue onto the next stage of their trajectory after having crossed the midline.

In the vertebrate forebrain and developing visual system, Slit can mediate both pre-crossing and post-crossing axon guidance, presumably by surrounding the pathway to shape its trajectory, consistent with a ‘surround repulsion’ model of guidance. Support for this model has been obtained from the Slit1/Slit2 knockout mice (Plump et al., 2002) and the zebrafish astray (Robo2) mutant (Hutson and Chien, 2002), both of which show multiple guidance errors at or near the chiasm. There is also evidence to suggest that Slit may act to channel axons navigating in the anterior (AC) and postoptic commissures (POC) across the rostral midline in the zebrafish forebrain (Barresi et al., 2005) and callosal axons across the cortical midline commissure in the mouse forebrain (Bagri et al., 2002; Shu et al., 2003). Taken together, the disparate modes by which Robo–Slit signaling guides commissural axons navigating in distinct regions of the nervous system support a context-specific role for these guidance molecules in the vertebrate CNS.

Much of our current understanding of the role of Robo–Slit signaling in vertebrate axon guidance has been obtained from analyses of commissural axon growth. However, the first axons in the brain establish longitudinal tracts (Chitnis and Kuwada, 1990). Longitudinal axons traverse relatively long distances to connect adjacent brain nuclei and allow connectivity between the rostral and caudal regions of the nervous system. Despite this, the mechanisms which direct the growth and guidance of axons in these longitudinal tracts remain poorly defined. Indeed, most studies of longitudinal axon guidance have focused only on the pathfinding behavior of commissural axons on the contralateral side of the floor plate in the spinal cord (reviewed in Stoeckli, 2006). The extent to which these principles may also be applicable in the context of ipsilateral projections in the brain is unknown.

In this study, we provide evidence that the Robo family of axon guidance receptors is necessary for the normal development of longitudinal axon tracts in the embryonic zebrafish brain. Using a loss-of-function approach, we demonstrate that Robo1, Robo2 and Robo3 have complementary functions in the establishment of the dorsoventral topology of the major longitudinal tract of the forebrain, the tract of the postoptic commissure (TPOC). Robo2 activity was essential to induce the normal defasciculation of the TPOC along the ventrolateral surface of the brain in response to the ligand Slit1a, while Robo3 and Robo1 acted to limit the dorsal spread of the TPOC axons. We propose that the complementary functions of Robo family members in the TPOC are necessary to position the trajectory of axons navigating within the major longitudinal axon tracts in the brain.

Materials and methods

Zebrafish

Fish were maintained at the University of Queensland SBMS fish facility on a 14-h light/10-h dark cycle. Embryos were staged as described (Kimelman, 1995). Embryos older than 24 h post-fertilization (hpf) were raised in 0.2 mM 1-phenyl-2-thio-urea (PTU; Sigma, St. Louis, MO) to prevent pigment formation.

In situ hybridization and immunohistochemistry

Probes were generated against robo1 (Challa et al., 2001), robo2, robo3 (Lee et al., 2001) and slitta (Hutson et al., 2003). It should be noted that two isoforms of robo3 have been described which differ in their 5′UTR and signal sequence (Challa et al., 2001; Lee et al., 2001). While the robo3 probe used here does not differentiate between these two forms, Challa et al. (2005) have demonstrated that both variants are expressed in the embryonic nervous system. Whole-mount in situ hybridization and HNK-1 immunohistochemistry were performed as previously described (Hjorth and Key, 2001). Gene expression patterns were visualized using Fast Red (Roche Ltd., Basel, Switzerland) as the chromogenic substrate.

Double-fluorescent in situ hybridization was performed essentially as previously described (Ramakrishnan, 2005). Fluorescein-labeled RNA probes (Roche) were detected using anti-fluorescein-AP (Roche) at 1:5000 and visualized using Fast Red. The reaction was stopped by washing in PBT (0.1 M PBS and 0.1% Tween-20). For the detection of DIG-labeled probe, embryos were incubated overnight in anti-DIG POD (1:1000, Roche) and visualized using Alexa Fluor® 488 conjugated TSA reagent (Molecular Probes, Eugene, OR). The reaction was performed for 1 h in the dark and stopped by washing in PBT.

Morpholino and RNA injections

Translation blocking morpholino oligonucleotides (MO) were purchased from Gene Tools LLC (Philomath, OR). Two non-overlapping antisense morpholino (MO) oligonucleotides were generated against the 5′ region for each of the genes used in this study. The data presented here relate to that obtained from one MO only (asterisk in Table 1). A standard control morpholino available from Gene Tools LLC was injected as one test for specificity. Morpholino sequences are listed in Table 1. Working dilutions (10 mg/ml) of each morpholino were prepared in water. Blastomeres at the 1-cell stage were injected with 1 nl of morpholino at a concentration of 5 ng/ml, with the exception of slitta MO, which was injected at 2.5 ng/ml. Embryos were raised to 28 hpf in embryo medium and fixed in 4% paraformaldehyde. Only those embryos which displayed a normal gross morphology were processed for subsequent analysis of the axon scaffold. This conservative approach excluded the possibility that axon guidance defects were generated indirectly by morphological defects such as shortening along the anteroposterior axis or abnormal cell death. It should be noted that most embryos developed normally after injection of robo1, robo2 or robo3var1 MOs (95.4%, 95% and 91% respectively). A previous study of Robo3 function in zebrafish described early patterning defects following knockdown of robo3var2 (Challa et al., 2005), while the robo3var2 MO used here failed to produce any gross morphological defect, despite being designed to...
Table 1

<table>
<thead>
<tr>
<th>Morpholino</th>
<th>Location in 5′ region</th>
<th>Sequence (5′ to 3′)</th>
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<tbody>
<tr>
<td>Robo1 MO1</td>
<td>−3 to +21</td>
<td>ATCCAAATTTACTCTCCCGGTCACTGG</td>
</tr>
<tr>
<td>Robo1 MO2</td>
<td>−13 to −37</td>
<td>CGGCTTTTTAGATAGCTGTTGTA</td>
</tr>
<tr>
<td>Robo2 MO1</td>
<td>−2 to +22</td>
<td>AAAAGGTGTGTTAAAGGACCACATCC</td>
</tr>
<tr>
<td>Robo2 MO2</td>
<td>−5 to −29</td>
<td>TCTATGTCACATCCACACCTCAGT</td>
</tr>
<tr>
<td>robo3var1 MO1</td>
<td>+1 to +26</td>
<td>GCATCCAAAGTCCTGCAGAAAACC</td>
</tr>
<tr>
<td>robo3var1 MO2</td>
<td>−14 to −39</td>
<td>AAGTCCACCTCTGTCGAAATTCA</td>
</tr>
<tr>
<td>Slit1a MO1</td>
<td>−3 to +21</td>
<td>TCTTTATCAGTTAAGGAGCAGCTCT</td>
</tr>
<tr>
<td>slit1a MO2</td>
<td>−5 to −29</td>
<td>TCTCTAAGACTCCCCGAGAATACTA</td>
</tr>
<tr>
<td>Control MO</td>
<td>−</td>
<td>CCTCTACCTCAGTTCAATTTATA</td>
</tr>
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</table>

* Denotes MO to which presented results correspond.

To begin to understand the role of members of the Robo family of axon guidance receptors in establishing the initial template of axon tracts in the developing vertebrate brain, we first examined the expression pattern of these genes by in situ hybridization. Between 16 and 24 hpf in zebrafish, a simple three-dimensional axon scaffold is established in the embryonic rostral brain, consisting of a stereotypical set of five axon tracts and four commissures (Ross et al., 1992; Wilson et al., 1990). The expression of robo1, -2, and -3 and their relationship to the developing axon scaffold was revealed in compiled, unilateral serial-confocal laser scans of whole-mount brains. These brains were also co-labeled with anti-HNK-1 to reveal postmitotic neurons and their axons (Metcalfe et al., 1990).

The principal longitudinal axon tract in the zebrafish forebrain, the postoptic commissure (TPOC) emerges from a ventrorostral cluster (vrc) of neurons. This cluster of neurons expresses all three Robos (Figs. 1A, C, E, dashed outline). Robo1 appeared to be expressed in an enlarged domain in the rostral half of the ventral diencephalon whereas Robo2 and 3 were more restricted to a ventral band that incorporated the whole vrc. As compiled confocal projections can sometimes give the false impression of co-localization of staining, single confocal scans were also individually examined. This analysis confirmed that each of the robos was expressed in the vrc (Figs. 1B, D and F, filled arrowheads). Double-fluorescent in situ hybridization was then used to determine the extent to which robo family members displayed overlapping distributions in the ventrorostral diencephalon. Single confocal scans revealed that robo1 co-localized with both robo2 (Fig. 1G, filled arrowheads) and robo3 (Fig. 1H, filled arrowheads) in a subset of cells that occupy a more rostral position (dashed outline in Figs. 1G and H demarcates this region of overlap). By contrast, the majority of the cells expressing robo2 were also found to co-express robo3 (Fig. 1I, filled arrowhead). robo3 was also detected in a number of cells surrounding those expressing robo2 (Fig. 1J, asterisk). These results demonstrate the heterogeneity of robo expression in the region encompassing the vrc and suggest the presence of neuronal subpopulations, each expressing a distinct complement of robo receptors: a rostrally located population that expresses all three robo family members and a caudally positioned population that expresses only robo2 and robo3 (Fig. 1J). Together, these results suggest that the Robo family of receptors may mediate the development of the TPOC in the embryonic vertebrate brain.

Knockdown of either Robo1 or 3 causes abnormal spreading of longitudinal axon tracts in the forebrain

To examine the role of each of the Robo family members in axon guidance in the rostral brain we knocked down gene function using antisense morpholin oligonucleotides (MOs). Two distinct non-overlapping MOs were generated against each of the three robo mRNAs. One of each pair of MOs was directed against 5′UTR sequence within 40 bases upstream of the start site, while the other incorporated the ATG start site (Table 1). In all cases, the phenotypes observed with the two independent MOs against each robo mRNA were similar, which confirmed the specificity of these knockdowns. For all MOs, a minimum of three independent repeats were performed.
**Fig. 1.** *Robo* family members are expressed in the brain during the formation of the axon scaffold. Lateral views of whole mount zebrafish brains double labeled for the expression of *robo* family members (red) and the HNK-1 epitope (green) at 22 h post-fertilization (hpf). Rostral is to the left and dorsal is to the top. (A) At 22 hpf, *robo1* expression is observed in the ventral diencephalon (dashed outline), ventrocaudal cluster (vcc) and midbrain. (C) *robo2* expression was observed in the ventrorostral cluster (vrc; dashed outline), dorsorostral cluster (drc) and vcc. (E) *robo3* expression was detected in the vrc (dashed outline), drc and vcc. Single slice confocal analysis of *robo* expression reveals co-localization of individual *robo* receptors to vrc cell bodies at 22 hpf (B, D and F, filled arrowheads). (G–I) Double in situ hybridization for *robo* family members in the rostral diencephalon at 22 hpf, rostral is to the left, dorsal is to the top. *robo1* co-localizes with *robo2* (G, filled arrowheads) and *robo3* (H, filled arrowhead) in a subset of rostrally positioned cells (dashed outline defines the caudal limit of co-expression). Outside of this region of overlap, additional cells were identified that expressed only one of the *robo* receptors (G and H, unfilled arrowheads). *robo3* co-localizes with the majority of *robo2*-expressing cells (I, filled arrowhead) and is also detected in surrounding cells (I, asterisk). (J) Schematic representation of *robo1*, *robo2* and *robo3* mRNA expression at 22 hpf. Scale bars: A, C, E: 50 μm; B, D, F: 10 μm; G–I: 12.5 μm.
Recorded defects for each repeat were subsequently compared using the Chi-squared test for homogeneity before pooling. The results presented here correspond to data obtained from one MO only (MO1 or MO2), as indicated. In embryos injected with the control MO (Fig. 2A), the axon tracts were indistinguishable from uninjected embryos.

In zebrafish, two isoforms of robo3 (robo3var1 and robo3-var2) have been identified which differ in their 5′UTR and signal sequence (Challa et al., 2001, 2005; Lee et al., 2001). Given the difference in their 5′ sequence, MOs were designed to specifically target each variant. Embryos injected with 5 ng of robo3var2 MO1 exhibited no significant defects in the development of their axon scaffold (compare Figs. 2A and B; Table 2). In contrast, 47% of embryos injected with 5 ng of robo3var1 MO1 displayed abnormal dispersion of fascicles within the caudal portion of the TPOC (Figs. 2C, E, arrowheads; Table 2). The TPOC courses from the rostral surface of the brain until its junction with the TPC. The axons in this tract typically

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![Fig. 2. Aberrant dorsal spreading of the TPOC is observed following knockdown of Robo3. Scaffolds of 28 hpf zebrafish embryos labeled with acetylated α-tubulin. Rostral is to the left, dorsal is to the top. (A) In control MO scaffolds (5 ng), the axon tracts develop normally. (B) No significant axon guidance defects were observed following knockdown of robo3var2. Aberrant growth of axons in the TPOC was observed in robo3var1 MO1-injected embryos; TPOC axons were observed to spread out into more dorsal regions of the mesencephalon (C, arrowhead; compare D and E). (F) Coinjection with 350 pg of robo3-rescue cRNA rescued the robo3var1 MO induced TPOC defect. (G–J) Robo3 morpholinos act specifically to knockdown their targeted isoform (var1 or var2). Embryos injected with 250 pg 5′robo3var1-GFP cRNA (G) or 250 pg 5′robo3var2-GFP cRNA (I) show strong fluorescence at 28 hpf, which is lost following coinjection with the respective MO (robo3var1 MO1, H or robo3var2 MO1, J). (I) robo3var1 MO1 failed to abrogate GFP expression when coinjected with 5′robo3var2-GFP cRNA, confirming that robo3-var1 MO1 specifically targets the var1 isoform. (K) The TPOC spreading phenotype was observed at a similar penetrance when both robo3var1 and robo3var2 were simultaneously knocked down (arrowhead). Scale bars: A–C, F, K: 50 μm; D, E: 17 μm; G–J: 375 μm.
As a further test for the specificity of the robo3 MOs, GFP fusion constructs were generated in which the 5′ regions of robo3var1 and robo3var2 were fused in frame to the coding region of enhanced green fluorescence protein (referred to here as 5′robo3var1-EGFP and 5′robo3var2-EGFP). Coinjection of 5′robo3var1-EGFP cRNA with the robo3var1 MO1 completely abrogated fluorescence (compare Fig. 2G with H). Similar results were obtained with robo3var2 MO1 after coinjection with 5′robo3var2-EGFP cRNA (Fig. 2J). These results indicated that the MOs were highly efficient in knocking down the expression of robo3var1 and robo3var2. Furthermore, the specificity of the robo3var1 MO1 was demonstrated by showing that it failed to block EGFP fluorescence after injecting the inappropriate 5′robo3var2-EGFP cRNA construct (Fig. 2I).

Next we knocked down the complete pool of robo3 by coinjecting 5 ng each of the robo3var1 and robo3var2 MOs (10 ng total MO). These animals were compared to those injected with 10 ng of control MO (Table 2). The defects observed in the axon scaffold of these robo3(var1+2) MO-injected animals were similar to those observed in the robo3var1 MO-injected embryos, confirming that Robo3var2 had little effect on axon growth in the TPOC (Fig. 2K, Table 2). This was also reflected in the average TPOC width for this group (Fig. 3H; 44.5±6.9 μm, n=12), which was not significantly different from that observed following knockdown of robo3var1 alone.

Next, embryos were injected with the robo1 MOs. Following injection of 5 ng robo1 MO2, 42% of scaffolds exhibited abnormal spreading of axons in the caudal TPOC (compare Fig. 3A with Figs. 3B and C, arrowheads, Table 2), as was observed following robo3 knockdown. Quantification of the TPOC spreading phenotype revealed that the width of the tract in robo1 knockdown scaffolds (Fig. 3H; 38.8±5.2 μm, n=16) was significantly greater than that observed in control MO scaffolds (Fig. 3H; 26.8±1.4 μm, n=16, P<0.001). We found that loss of Robo3 had no effect on the mediolateral positioning of axons. Defects were instead restricted to the dorsosrenal axis.

To confirm that the MO1 phenotypes were generated by specific knockdown of robo3var1, we carried out a rescue experiment. In order to perform this experiment we generated a robo3var1 variant cDNA which lacked the 5′ region recognized by MO1. This was achieved by replacing the first 22 amino acids of robo3var1 (which contained the region of the mRNA recognized by the MO) with a generic IgK-membrane targeting sequence (referred to as robo3-rescue). Expression of robo3-rescue cRNA by injection into I-cell embryos produced the same gross morphological abnormalities (for example, shortening of the trunk) as overexpression of native robo3var1 cRNA (data not shown) which confirmed that the fusion protein was functionally active. Next we coinjected 5 ng robo3var1 MO1 together with 350 pg robo3-rescue cRNA in an attempt to rescue the mutant phenotype. The expression of the robo3-rescue was sufficient to significantly reduce the penetrance of the TPOC spreading defect from 47% down to 12% (Fig. 2F; n=51, P<0.0001). The ability of robo3-rescue to specifically rescue the MO phenotype confirmed that the axon guidance defects were a result of the selective loss of Robo3var1 and provided additional confirmation of the specificity of the robo3var1 MOs.

course as a single bundle until they reach the level of the DVDT, where they split into several smaller fascicles (Fig. 2D). Loss of robo3var1 caused these fascicles to splay out over the ventral surface of the mesencephalon (Fig. 2E, arrowheads). The extent of TPOC spreading in these scaffolds was quantified by measuring the width of the TPOC along its dorsoventral axis (45.2±5.6 μm, n=10) and found to be significantly greater than that of control MO scaffolds (Fig. 3H; 38.8±5.2 μm, n=16) (Table 2). We found that loss of Robo3 had no effect on the mediolateral positioning of axons. Defects were instead restricted to the dorsosrenal axis.

Table 2
Axon guidance defects following knock down of Robo receptors

<table>
<thead>
<tr>
<th>Scaffolds analyzed</th>
<th>TPOC spreading</th>
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<tbody>
<tr>
<td>5 ng Control MO</td>
<td>0</td>
</tr>
<tr>
<td>10 ng Control MO</td>
<td>0</td>
</tr>
<tr>
<td>15 ng Control MO</td>
<td>0</td>
</tr>
<tr>
<td>5 ng robo1 MO2</td>
<td>42% (20)</td>
</tr>
<tr>
<td>5 ng robo2 MO2</td>
<td>0</td>
</tr>
<tr>
<td>5 ng robo3var2 MO1</td>
<td>0</td>
</tr>
<tr>
<td>5 ng robo3 var1 MO1</td>
<td>73% (34)</td>
</tr>
<tr>
<td>5 ng robo3var1 MO1+5 ng robo3var2 MO1</td>
<td>56% (25)</td>
</tr>
<tr>
<td>5 ng robo1 MO2+5 ng robo3var1</td>
<td>70% (54)</td>
</tr>
</tbody>
</table>

a Numbers in parentheses depict the number of scaffolds.

b Significantly different (p<0.0001) from 5 ng control MO-injected embryos.

c Significantly different (p<0.0001) from 10 ng control MO-injected embryos.

d Significantly different (p<0.0001) from 10 ng rob3 MO-injected embryos.

e Significantly different (p<0.0001) from 5 ng robo1 MO-injected embryos.

f Significantly different (p<0.05) from 5 ng robo1 MO-injected embryos.

As a further test for the specificity of the robo3 MOs, GFP fusion constructs were generated in which the 5′ regions of robo3var1 and robo3var2 were fused in frame to the coding region of enhanced green fluorescence protein (referred to here as 5′robo3var1-EGFP and 5′robo3var2-EGFP). Coinjection of 5′robo3var1-EGFP cRNA with the robo3var1 MO1 completely abrogated fluorescence (compare Fig. 2G with H). Similar results were obtained with robo3var2 MO1 after coinjection with 5′robo3var2-EGFP cRNA (Fig. 2J). These results indicated that the MOs were highly efficient in knocking down the expression of robo3var1 and robo3var2. Furthermore, the specificity of the robo3var1 MO1 was demonstrated by showing that it failed to block EGFP fluorescence after injecting the inappropriate 5′robo3var2-EGFP cRNA construct (Fig. 2I).

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Robo1 and Robo3 act cooperatively in the TPOC

Our results above revealed that both Robo1 and Robo3 were involved in maintaining longitudinally projecting axons within the normal dorsosrenal limits of the TPOC. Next we examined whether Robo1 and Robo3 acted cooperatively to direct the
guidance of axons navigating in the TPOC. We began to address this question by simultaneously knocking down both robo1 and robo3 (both var1 and var2) by coinjection of 5 ng robo1 MO2, TPOC axons were observed to spread inappropriately into more dorsal regions of the mesencephalon (B and C; arrowheads). (D–G) The specificity of the robo1 MO knockdown was verified by loss of GFP fluorescence following coinjection of 250 pg 5′robo1-GFP cRNA and 5 ng robo1 MO2 (E, bright field in panel F) and rescue of the TPOC phenotype by coinjection with 250 pg of robo1-rescue cRNA (G). (H) The severity of the TPOC spreading phenotype was quantified by measuring the width of the TPOC. Values represent the mean±SD. Average TPOC width measurements were analyzed using ANOVA, followed by the Tukey HSD test, *P<0.05, **P<0.001. Scale bars: A, B, G: 50 μm; C: 20 μm; D–F: 400 μm.

Knockdown of robo2 leads to partial collapse of the TPOC

We next examined if Robo2 may also play a role in the guidance of axons in the TPOC by knocking down its function using two independent MOs. We chose a morpholino-based approach because of the ease in regulating the extent of Robo2 knockdown (by titering the amount of morpholino injected) and because it is not, as yet, clear whether the astray mutant (Fricke et al., 2001) is a gain-of-function or loss-of-function mutation. Since both MOs produced similar results, the data presented here were obtained using robo2 MO2. The most striking abnormality was the reduced dorsoventral width of the TPOC caudal to the DVDT-TPOC intersection (compare Fig. 4A with B, and C and D, arrowheads). The increased significant penetrance of the TPOC phenotype following the simultaneous knockdown of robo1 and robo3, as compared to either robo1 or robo3 separately (~42–45%; Table 2), confirmed that these receptors had similar roles and acted cooperatively in this pathway.

Fig. 3. Aberrant dorsal spreading of the TPOC is observed following knockdown of Robo1. Dissected brains of 28 hpf zebrafish embryos labeled with acetylated α-tubulin to show all axons. Rostral is to the left and dorsal is to the top. (A) In control embryos the scaffold of axon tracts develops normally. Following injection of 5 ng robo1 MO2, TPOC axons were observed to spread inappropriately into more dorsal regions of the mesencephalon (B and C; arrowheads). (D–G) The specificity of the robo1 MO knockdown was verified by loss of GFP fluorescence following coinjection of 250 pg 5′robo1-GFP cRNA and 5 ng robo1 MO2 (E, bright field in panel F) and rescue of the TPOC phenotype by coinjection with 250 pg of robo1-rescue cRNA (G). (H) The severity of the TPOC spreading phenotype was quantified by measuring the width of the TPOC. Values represent the mean±SD. Average TPOC width measurements were analyzed using ANOVA, followed by the Tukey HSD test, *P<0.05, **P<0.001. Scale bars: A, B, G: 50 μm; C: 20 μm; D–F: 400 μm.
surface area of the vrc and examined the early development of this tract. We found no change in the size of the vrc as defined by the expression of HuC, a postmitotic neuronal marker. The surface area of the vrc in embryos injected with 5 ng \textit{robo2} MO (1655±85 μm², n=15) was not significantly different from that of embryos injected with 5 ng control MO (1682±103 μm², n=15, P=0.22). There was also no qualitative difference in the formation of the TPOC at 18–20 hpf (when pioneer axons establish this tract) in embryos injected with either 5 ng \textit{robo2} MO (Fig. 4G; 100%, n=10) or 5 ng control MO (Fig. 4F, 100%, n=16). Thus, \textit{Robo2} clearly has a role in maintaining the normal fasciculation of the TPOC in the caudoventral diencephalon.

\textbf{Robo–Slit activity is necessary to maintain the dorsoventral topology of the TPOC}

To better understand how the Robo receptor family controlled the trajectory of axons in the TPOC, we next examined the role of its chemorepulsive ligand Slit. If the effects of Robo are mediated by binding to Slit, it follows that the topology of this tract observed following knockdown of \textit{slit} should phenocopy that seen in \textit{robo} loss-of-function scaffolds. Four Slit family members have been identified from zebrafish: \textit{slit1a}, \textit{slit1b}, \textit{slit2} and \textit{slit3} (Hutson et al., 2003; Yeo et al., 2001). \textit{Slit1a} was of particular interest in the context of the development of the TPOC due to its prominent expression in the ventral diencephalon and mesencephalon (Hutson et al., 2003). The remaining Slit family members were not considered to be strong candidates to influence the development of this tract because their expression is restricted to the rostroventral midline rather than exhibiting a sharp boundary as for \textit{slit1a} within the pathway of the TPOC (Barresi et al., 2005; Yeo et al., 2001).

We first sought to determine if the spatiotemporal expression of \textit{slit1a} was consistent with a role in the guidance of axons navigating in the TPOC. The expression pattern of \textit{slit1a}, and its relationship to the developing axon scaffold was examined in whole mounts of zebrafish brains at 18 and 22 hpf by in situ hybridization. These brains were also co-labeled with anti-HNK-1 to reveal postmitotic neurons and their axons. At 18–20 hpf, when axons arising from the vrc are beginning to pioneer the TPOC (Ross et al., 1992), \textit{slit1a} was expressed in the ventral brain and midline of the caudal diencephalon and mesencephalon (Fig. 5A, dashed line). The rostral limit of \textit{slit1a} expression was
positioned approximately midway between the vrc and vcc (Fig. 5A). By 22 hpf, axons from the vrc had extended caudally and begun to enter the neuroepithelial domain expressing slit1a (Figs. 5B, C). As the single bundle of axons in the TPOC entered this slit1a domain it defasciculated into several separate fascicles.

Knockdown of slit1a caused a significant reduction in the width of the TPOC (compare Fig. 5D with E; 17.6±3.1 μm, n=19, P<0.01) which resembled the TPOC phenotype obtained following knockdown of robo2 (Fig. 5F). These results suggested that Robo2 was acting through Slit1a to maintain the normal width of the TPOC and that loss of either one caused its partial collapse. To test whether Robo2 and Slit1a were acting in the same ligand–receptor pathway in the TPOC, we next investigated potential in vivo genetic interactions using a MO-based strategy which parallels either Drosophila transheterozygote experiments (Kidd et al., 1999) or dual knockdown approaches in Xenopus (Wills et al., 2006; Wilson and Key, 2006) and zebrafish (Feldner et al., 2007; Solomon et al., 2004). We predicted that if Slit1a was acting through Robo2 in the TPOC, then coinjection of a subthreshold concentration of robo2 MO would enhance the weak TPOC collapse phenotype generated by partial knockdown of slit1a MO. A subthreshold concentration of robo2 MO was identified (2 ng) at which the width of TPOC in injected scaffolds (Fig. 5I, 23.5±2.7 μm, n=33) was not significantly different from that observed following injection of control MO (Fig. 5I, 25.4±2.1 μm, n=15). Upon coinjection of 2 ng robo2 MO with a reduced concentration of slit1a MO (1 ng), a more complete collapse of the TPOC was observed (Figs. 5G, I; 10.4±2.4 μm, n=36). In these animals the axons in the TPOC now appeared to course as a single thick bundle between the DVDT and TPC instead of as ~4 distinct fascicles. The enhanced phenotype obtained following partial knockdown of both robo2 and slit1a...
suggests that coinjection both MOs reduce function more completely than each alone. These results support the idea that Robo2 and Slit1a are both acting to maintain the normal spread of fascicles in the caudal portion of the TPOC. Thus these results are consistent with the model whereby Robo2-expressing axons defasciculate when they confront a domain of chemorepulsive Slit1a.

We demonstrated above that knockdown of either robo1 or robo3 resulted in the aberrant dorsal spreading of the TPOC. In contrast, loss of Slit1a resulted in the collapse of this tract. To understand the interaction of these phenotypes we simultaneously knocked down both robo3 and slit1a. This double knockdown resulted in a slit1a-like phenotype (Fig. 5H). There was a significant loss of the TPOC spreading defect (from 45% down to 6%; P<0.0001). Similar results were also obtained when robo1 and slit1a MOs were coinjected (not shown). The predominance of the slit1a phenotype in the absence of either Robo3 or 1 is consistent with Slit1a acting earlier in the development of the TPOC than either of these two Robos. These results support the model whereby Robo2–Slit1a interactions initially defasciculated the tract and then later, Robo3 and 1 act to prevent further spreading.

Next we examined double knockdowns of robo2 and either robo1 or robo3. The knockdown of robo1 or robo3 was capable of significantly rescuing the TPOC collapse phenotype that was associated with knockdown of robo2 (Table 2). The average width of the TPOC following knockdown of robo2 and either robo1 (27.7 μm, n=18) or robo3 (both variants; 23.5 μm, n=20) was not significantly different from control MO samples. This rescue suggests that Robo1 and 3 are, at least, partially responsible for the increased fasciculation in the absence of Robo2. These results are consistent with a model whereby Robo2–Slit1a interactions initially caused defasciculation of the TPOC and then the presence of Robo1 and 3 limited the extent of this defasciculation.

In summary, these results demonstrate that Robo1 and Robo3 prevent axons from straying into inappropriate dorsal regions of the diencephalon and mesencephalon, whereas Slit1a–Robo2 signaling is necessary to induce the normal spreading of the TPOC caudal to its junction with the DVDT. Thus, the roles of Robo1 and Robo3 are complementary to that of Robo2. Robo1 and Robo3 act to limit the spread of axons and appear to counterbalance the defasciculating role of Robo2.

Discussion

The Robo family of chemorepulsive receptors have a clear role in the guidance of commissural axons across the midline that is conserved across species (Long et al., 2004; Rajagopalan et al., 2000a; Sabatier et al., 2004). In the present study, we show for the first time that these guidance molecules are also involved in the development of the longitudinal axon tracts in the embryonic vertebrate brain. Unlike spinal cord commissural axons that course into, and out of, a narrow band of midline glial cells expressing Slit (Brose et al., 1999; Long et al., 2004), we show that pioneer Robo-expressing longitudinal axons arising in the rostral diencephalon course continuously within a broad domain of cells expressing Slit1a in the caudal diencephalon (Fig. 6A). Three members of the Robo family (Robo1, Robo2 and Robo3) act co-ordinately to ensure that these longitudinally growing axons establish the normal topology of the tract of the postoptic commissure (TPOC) within this Slit1a domain.

The TPOC is the major longitudinal tract in the embryonic vertebrate forebrain and its temporal and spatial development has been studied in zebrafish (Chitnis and Kuwada, 1990; Wilson et al., 1990), chick (Chedotal et al., 1995) and rodents (Andrews and Mastick, 2003; Mastick and Easter, 1996). The TPOC courses caudally through the ventral diencephalon, merging with longitudinal tracts in the mesencephalon and rhombencephalon. Axon fascicles contributing to this tract display different degrees of fasciculation along its length. In the rostroventral diencephalon, the TPOC is a tightly fasciculated axon bundle, whereas more caudally, it splits into a number of distinct parallel fascicles which are spread across the ventro-caudal surface of the diencephalon. Conservation of the organization of these axons in the evolutionary distant zebrafish and mouse may be related to the role this tract plays as a template for the pathfinding of other axons (Chitnis and Kuwada, 1991; Wilson and Easter, 1991). In zebrafish, the TPOC arises initially from a ventrostral cluster of neurons, the vrc, which expresses all three robo genes (robo1, robo2 and robo3) by 22 hpf. Our in situ hybridization analysis revealed the existence of two subpopulations of vrc neurons; a rostral cluster of neurons expressing robo1, robo2 and robo3, as well as a caudal cluster expressing only robo2 and robo3 (Fig. 1J). The axons of these neurons initially course in a thick bundle until approximately midway between the vrc and the ventro-caudal cluster of neurons (vcc), where the TPOC disperses into several discrete fascicles. Since the separation of this tract coincided with the point at which the axons enter a broad ventral domain of neuroepithelium expressing slit1a (Fig. 6A), it suggested that this ligand may be regulating the dispersion of axons.

Slit1a-dependent Robo2 activity directs the dispersion of longitudinal axons in the embryonic forebrain

Using a loss-of-function strategy, we demonstrated that Robo2 controlled the initial splitting of the TPOC into discrete fascicles as it entered a neuroepithelial domain expressing slit1a in the ventrocaudal diencephalon. In the absence of Robo2, the axon fascicles in this tract collapsed within the slit1a-expressing region (Fig. 6B). The role of Slit1a in directing this behavior was confirmed by knocking down its expression, which resulted in a similar collapsed phenotype. The simplest interpretation of this behavior is that TPOC axons are initially dispersed by the chemorepulsive activity of Slit1a but still continue growing longitudinally within smaller fascicles. This is consistent with previous reports that axons can grow into regions expressing chemorepulsive ligands. In the chick tectum, retinal axons expressing the EphA3 receptor initially overshoot their termination zone and grow over a repulsive substrate of ephrinA. While this repulsive gradient is adequate to prevent branching along the axon, it is not sufficient to prevent growth.
cone advance (Yates et al., 2001). There is also recent evidence that modifying molecules can dampen the chemorepulsive response of axons to Slit. Stromal cell-derived factor-1 (SDF1) has been implicated as an important modulator of Robo–Slit signaling in the zebrafish optic tract. SDF-1 is capable of reducing the response of retinal axons to Slit2, allowing these axons to extend in the presence of this chemorepellent (Chalasani et al., 2007).

The genetic interaction we observed between Slit1a and Robo2 in the TPOC is consistent with recent evidence that Slit1a was also acting through Robo–Slit signaling in the other regions of the nervous system (Campbell et al., 2007; Cho et al., 2007). Our results do not provide an insight into whether Slit1a is necessary for the action of Robo1 and Robo3 in the TPOC. They do however suggest that Slit1a is not directly responsible for the function of these Robos since the Slit1a and Robo1/3 knockdown phenotypes are distinct. If Slit1a is involved in the role of Robo1/3 in limiting the spread of axons then these interactions must be indirect and modulated by other proteins.

The collapse of the TPOC along its dorsoventral axis following knockdown of Robo2 is reminiscent of Robo mutant phenotypes in Drosophila, where a ‘Robo code’ has been demonstrated to be necessary for the correct formation of the longitudinal axon tracts (Simpson et al., 2000a). In Drosophila, axons upregulate their expression of Robo, Robo2 and Robo3 after crossing the midline. This not only prevents axons recrossing the midline but also appears to dictate their lateral position within the three distinct fasciclin II expressing axon bundles present on either side of the Drosophila midline (Rajagopalan et al., 2000b; Simpson et al., 2000a). The specific combination of Robo receptors expressed by an axon will determine its sensitivity to the gradient of Slit emanating from the midline, and consequently, its lateral position in the longitudinal pathways. In these animals, loss of Robo2 or Robo3 resulted in the collapse of axon fascicles into fewer bundles, presumably because they experience less chemorepulsion from the midline. Our results suggest that Slit-mediated repulsion of Robo-positive axons, as a general mechanism for patterning the longitudinal axon tracts, appears to have been conserved in both Drosophila and vertebrates. However, unlike Drosophila where the longitudinal tracts are organized along the mediolateral plane parallel to the midline, in vertebrates, the longitudinal tracts are organized along the dorsoventral axis, perpendicular to the midline. Accordingly, in the zebrafish forebrain, it is not the expression of Slit at the midline which is integral to establishing the dorsoventral polarity of the TPOC, but the presence of Slit1a in the neuroepithelium through which these axons must navigate. While our analysis of robo expression in the rostrocaudal diencephalon identified a number of subpopulations which each expresses a unique combination

Fig. 6. Robo family members have complementary roles in the TPOC (A) Slit1a–Robo2 interaction is necessary to direct the dorsoventral positioning of axons in the TPOC. Axons navigating in the TPOC initially grow out in a tightly fasciculated axon bundle. Upon reaching a wall of slit1a expression, Robo2-mediated chemorepulsion is sufficient to split the TPOC from a single bundle of axons into 4–6 distinct fascicles. (B) Knockdown of robo2 and slit1a function causes axons to remain in a tightly fasciculated bundle due to an inability to detect the repulsive cue. (C) By contrast, Robo1 and Robo3 act to prevent TPOC axons from coursing inappropriately into dorsal regions of the ventrocaudal diencephalon. Consequently, increased spreading of the TPOC is observed in the absence of Robo1 or Robo3. (D) Taken together, the normal topology of the TPOC is achieved through the complementary roles of Robo2-dependent spreading in response to Slit1a (red arrows) and the subsequent dampening effects of Robo3 and Robo1 (blue arrows).
of robo receptors, a more detailed understanding of the extent to which this co-expression may translate into a functional ‘Robo code’ in the TPOC will require the identification of more appropriate markers that clearly delineate axonal populations.

In the vertebrate spinal cord, there is some evidence to suggest that Robo–Slit interactions may play a role in determining the relative lateral positions at which pre-crossing commissural axons turn into longitudinal pathways (Long et al., 2004). Axons expressing Robo1 appear to be expelled only a short distance from the floor plate, whereas those expressing Robo2 are driven farther from the floor plate into more dorsolateral regions of the spinal cord, presumably due to a heightened sensitivity of Robo2 to Slit. In Robo2 null mice the lateral funiculus is reduced in its spread across the lateral surface of the spinal cord (Long et al., 2004). While these results are consistent with the role of Robo2 in the TPOC, it was not clear from this study whether defects observed in the relative positions to which commissural axons grew were directly attributable to reduced Slit-dependent chemorepulsion. Although the lateral funiculus did not spread as far dorsally in Slit mutants this may have been a result of perturbed midline crossing. In the absence of commissural defects, we have been able to demonstrate that Robo2–Slit1a interactions are directly responsible for regulating the spreading of the TPOC.

Robo1 and Robo3 limit the spreading of longitudinal axons

While robo2 loss-of-function brought about the collapse of the TPOC along its dorsoventral axis, knockdown of either robo1 or robo3 resulted in increased spreading of the TPOC. These complementary roles are necessary for the normal development of the TPOC. Interestingly, our results reveal an important temporal aspect to Robo activity in this tract; Robo2 activity is required initially to cause spreading of the TPOC in response to Slit1a, while Robo3 and Robo1 act sequentially to restrict this spreading. How might the complementary roles of these Robo family members converge to control the spreading of axons in this tract? A number of possible scenarios can be envisaged. For instance, there is evidence to suggest that Robo family members may regulate the activity of one another, either by direct or indirect means (Sabatier et al., 2004). Using double in situ hybridization, we demonstrated that robo family members displayed co-localization in the rostrocaudal diencephalon. While robo1 was only found to co-localize with robo2 and robo3 in the rostral vrc, robo2 and robo3 demonstrated a high degree of cellular co-localization throughout this nucleus. Robo3 may therefore act cell autonomously to modulate Robo2 function in the TPOC, analogous to the situation reported in the mouse spinal cord where Rig-1/Robo3 regulates Robo1 activity in pre-crossing commissural axons (Sabatier et al., 2004). Exactly how Robo3 and Robo1 could modulate Robo2 function is not clear, just as the mechanism underlying the proposed masking of Robo1 by Rig-1 remains unknown (Sabatier et al., 2004). While this interaction could be direct, it may also involve the convergence of downstream signaling pathways and the presence of accessory proteins specific to this trajectory.

Alternatively, Robo1 and Robo3 may function in a non-cell autonomous capacity to guide longitudinal projecting axons. In vitro, Robo1 and Robo2 can mediate homophilic and heterophilic binding, an interaction which can promote axon outgrowth (Hivert et al., 2002). Recently, Robo3 has been shown to also bind homophilically, however it does so at a lower efficiency than it binds heterophilically to either Robo1 or NCAM (Camurri et al., 2005). Thus, the collapse of the TPOC observed in the absence of Robo2–Slit1a interactions may be accounted for by increased axon–axon adhesion mediated in part by Robo1 and Robo3. Likewise, a reduction in adhesion would also explain the increased spreading of the TPOC in the absence of either Robo1 or Robo3. Further work will be necessary to characterize the precise function of Robo1 and Robo3 in the TPOC, however a more detailed analysis will first require the identification of more appropriate markers for axonal subpopulations.

The results presented here support a model in which Slit1a-dependent Robo2 activity is necessary to direct the dorsoventral positioning of longitudinal axon tracts in the TPOC. Remarkably, this activity is complementary to the functions of both Robo1 and Robo3, which act subsequently to prevent TPOC axons from coursing inappropriately into dorsal regions of the ventrocaudal diencephalon. Therefore, the normal trajectory of axons in the TPOC relies on the interplay of Robo2-dependent spreading of the TPOC upon exposure to Slit1a and the dampening effects of Robo3 and Robo1 (Fig. 6D). Taken together, our results highlight the context-specific behavior of guidance molecules in regulating the development of axonal tracts.

Acknowledgments

The authors would like to acknowledge Robin Connor for the design of the Robo3 morpholinos used in this study and Christina Claxton for the valuable advice on constructs. This work was supported by an Australian Research Council grant to B.K. and an Australian Postgraduate Award to C.A.D.

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