



## Robos are required for the correct targeting of retinal ganglion cell axons in the visual pathway of the brain

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Received 20 August 2007; revised 24 November 2007; accepted 12 December 2007 Available online 23 December 2007

Axonal projections from the retina to the brain are regulated by molecules including the Slit family of ligands [Thompson, H., Barker, D., Camand, O., Erskine, L., 2006a. Slits contribute to the guidance of retinal ganglion cell axons in the mammalian optic tract. Dev. Biol. 296, 476-484, Thompson, H., Camand, O., Barker, D., Erskine, L., 2006b. Slit proteins regulate distinct aspects of retinal ganglion cell axon guidance within dorsal and ventral retina. J. Neurosci. 26, 8082-8091]. However, the roles of Slit receptors in mammals, (termed Robos), have not been investigated in visual system development. Here we examined Robo1 and 2 mutant mice and found that Robos regulate the correct targeting of retinal ganglion cell (RGC) axons along the entire visual projection. We noted aberrant projections of RGC axons into the cerebral cortex, an area not normally targeted by RGC axons. The optic chiasm was expanded along the rostro-caudal axis (similar to Slit mutant mice, Plump, A.S., Erskine, L., Sabatier, C., Brose, K., Epstein, C.J., Goodman, C.S., Mason, C.A., Tessier-Lavigne, M., 2002. Slit1 and Slit2 cooperate to prevent premature midline crossing of retinal axons in the mouse visual system. Neuron 33, 219-232), with ectopic crossing points, and some axons projecting caudally toward the corticospinal tract. Further, we found that axons exuberantly projected into the diencephalon. These defects were more pronounced in Robo2

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Available online on ScienceDirect (www.sciencedirect.com).

than Robo1 knockout animals, implicating Robo2 as the predominant Robo receptor in visual system development. © 2007 Elsevier Inc. All rights reserved.

Keywords: Slit; Optic chiasm; Axon guidance; Retinal ganglion cells; Diencephalon

### Introduction

The Slit family of inhibitory molecules are crucial for the formation of the projections from the eye to the brain (Erskine et al., 2000; Niclou et al., 2000; Plump et al., 2002; Ringstedt et al., 2000). In Slit1/Slit2 double knockout mice, the optic chiasm is expanded in the rostro-caudal axis, suggesting that Slits are involved in defining the position and boundaries of the optic chiasm (Plump et al., 2002). More recent experiments show that Slits regulate axon pathfinding along the entire tract (Thompson et al., 2006a,b).

Genetic and biochemical evidence has demonstrated that Slit is a ligand for Roundabout (Robo) (Battye et al., 1999; Brose et al., 1999; Kidd et al., 1999; Li et al., 1999). To date, four mammalian Robo homologues have been identified. Three of these, Robo1, Robo2 and Robo3 (Rig1), are expressed within the developing nervous system (Kidd et al., 1998; Marillat et al., 2002; Sundaresan et al., 1998; Yuan et al., 1999). However, it is currently not known which of these receptors in mammals is likely to mediate Slit signaling to regulate visual system development. Experiments in zebrafish have shown that Robo2 (astray) mutants have defects in retinal axon pathfinding (Fricke et al., 2001) and that Robo2 acts to shape their pathway by both preventing and correcting pathfinding errors (Hutson and Chien, 2002). New data suggests that Robo2 also usually prevents the arborization and synaptogenesis of retinal ganglion cell (RGC) axons

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<sup>1044-7431/\$ -</sup> see front matter  $\ensuremath{\mathbb{C}}$  2007 Elsevier Inc. All rights reserved. doi:10.1016/j.mcn.2007.12.017

(Campbell et al., 2007) and Robo1 haplo-insufficiency has been implicated in human dyslexia (Hannula-Jouppi et al., 2005).

Recently Robo1, Robo2 and Robo3 mutant mice were generated by gene-trap technology (Robo1 and Robo2) and gene-targeting (Robo3) (Long et al., 2004; Sabatier et al., 2004) and shown to regulate the correct pathfinding of spinal commissural axons as they approach and cross the midline of the spinal cord. Gene-trap Robo1 and Robo2 mutant mice are reported to be severe hypomorphs (Long et al., 2004) or complete nulls (Grieshammer et al., 2004) respectively. In the brain, single mutants for either Robo1 and Robo2 have subtle defects and the two genes appear largely redundant (Lopez-Bendito et al., 2007). Double knockouts of these lines showed that both Robo1/2 are required for the formation of the major projections from cortex and thalamus (Lopez-Bendito et al., 2007) and the lateral olfactory tract (Fouquet et al., 2007).

Here we examine the phenotype of the visual system projections in Robo1 and Robo2 knockout mice that are different from those described above and generated by gene-targeting. We previously showed that even as single knockouts, these Robo1 knockout mice (but not Robo2 knockout mice) have severe defects in cortical and thalamic axonal projections as well as interneuron migration defects (Andrews et al., 2006). However, here we show that in the visual system, Robo2 is the predominant gene required, with Robo1 playing a more minor role in RGC axon targeting. The defects in each case are evident as single knockouts of each gene, and the brain and visual system defects observed indicate that these genes are not redundant in these systems but play specific and essential roles in their development.

### Results

## Expression of Robo1 and Robo2 proteins in the retina and visual pathway

We previously investigated Robo protein expression in the visual system using a pan Robo antibody (Sundaresan et al., 2004), but this did not allow us to distinguish between the expression of these two receptors. The expression of Robo1 and Robo2 were distinguished using Robo1 and Robo2 specific antibodies (Andrews et al., 2006; Long et al., 2004; Sabatier et al., 2004) in the retina and optic tract. At E14 and E12 respectively, Robo1 and Robo2 were first expressed in the retina and optic nerve head as the RGC axons projected from the retina (Figs. 1A, B). By E14, Robo1 and Robo2 were expressed in the optic chiasm (Figs. 1C and D arrowheads and arrowheads in G and H at E18) and along the post chiasmatic tract. From E15 to E18, both Robo1 and Robo2 were expressed in RGC axons as they grow over the diencephalon (Figs. 1E, F- shown at E18) and Robo2 was strongly expressed as they entered the superior colliculus (Fig. 1H large arrows). This data indicated that both Robo1 and 2 are expressed along the entire optic tract, but that Robo2 is expressed earlier than Robo1. Robo2 was more strongly expressed than Robo1 in the optic nerve and in the optic chiasm, but both were equally expressed on the distal regions of the tract. Thus, based on this expression analysis, one or both of these receptors are likely to be involved in the development and guidance of RGC axons, but it is not known whether these receptors are co-expressed in the same neurons.

## Abnormal development of the optic chiasm in Robo2 deficient mice

In order to assess the role of Robo1 and Robo2 in axon guidance at the optic chiasm, we performed both tract tracing studies and



Fig. 1. Expression of Robo1 and Robo2 in the visual projection. Wildtype mouse brain sections from E12-E18 were stained with Robo1 (A, C, E and G) and Robo2 (B, D, F and H) specific antibodies. At early stages of development Robo1 and Robo2 were expressed in RGC cell axons within the retina and the optic nerve. Robo2 was first expressed at E12 (arrow in B, horizontal section) whereas Robo1 began to be expressed at E14 in the optic nerve (arrow in A, horizontal section). At E14 both Robos were expressed at the optic chiasm, but Robo2 was more strongly expressed (arrowhead in C and D, horizontal sections). At E18 both Robos are expressed in the optic tract overlying the diencephalon (arrows in E and F, coronal sections), and in the optic chiasm (arrowheads in G and H; sagittal sections). Also visible by sagittal view is expression of Robo2 in the superior colliculus (large arrow in H) and the corticospinal projection that expresses both Robo1 and 2 (arrows in G and H). Scale bar in D=250 µm in A, 200 µm in B and 500 µm in C-F; bar in H=500 µm in G, H. Orientation in C is for panels A, B, C and D; orientations in E and G are for panels E, F and G, H, respectively (R, rostral, C, caudal, D, dorsal, V, ventral).

immunohistochemistry on Robo knockout brains. We previously described the generation of these Robo2 mutant mice (Andrews et al., 2008; Lu et al 2007), and in the case of the Robo1 mutant formerly demonstrated that these mice express neither Robo1 mRNA or protein, and can thus be considered true nulls. Here we also demonstrate that the Robo2 mutants are indeed true nulls, as we failed to detect the presence of Robo2 mRNA or protein in mutant

animals, which was present in control littermates (see supplemental Fig. 1).

Eyes of Robo1 or Robo2 deficient mice (Andrews et al., 2006; Lu et al., 2007) were injected with either DiI or DiA that was allowed to transport for 1–2 weeks before analysis. A ventral view of the optic chiasm is shown in Fig. 2 where Robo1–/– (n=6; Fig. 2C) and Robo2–/– animals (n=12; Fig. 2A) at E18 are compared with Robo2 (+/–; n=12; Fig. 2B). In Robo2–/– mice, we found three primary defects: 1) the chiasm appeared wider in the rostro-caudal dimension

(Figs. 2A, J and M); 2) some axons projected into the contralateral nerve (Fig. 2A arrow and yellow labelled axons); and 3) an ectopic caudal projection was evident which in some cases had lateral branches projecting from it (Figs. 2D, G, and H). This expansion of the optic chiasm in the rostro-caudal dimension is similar to the phenotype of the Slit1 and Slit2 double knockout mice (Plump et al., 2002), but such defects were not observed in Slit single mutants.

To quantify this expansion, we measured the medio-lateral width and the rostro-caudal length of the optic chiasm in Robo1 and Robo2



Fig. 2. Robo2 knockout mice display defects at the optic chiasm. The optic chiasm was visualized by filling eyes with DiI and DiA at E18 (A–C, ventral views; K–M, horizontal sections), or DiI only at E16 (D, G and H, horizontal sections), or with neurofilament (3A10) immunostaining at E16 (E, F, I and J, horizontal sections). In DiI/DiA images of Robo2 knockout mice axons from one eye enter the optic nerve and grow towards the contralateral eye (yellow axons at arrow in A) but do not in Robo2 heterozygotes (B) or Robo1 knockouts (C). At the optic chiasm some axons displayed an ectopic caudal projection (D, G, H, DiI only images at E16; D and G/H are two different examples, H is a higher-powered view of the boxed region in G). Dye tracing (A–C) and 3A10 immunohistochemistry at E16 (E, F, I, J) reveal an expansion of the optic chiasm in both the rostro-caudal and medio-lateral dimensions in Robo2 knockouts compared to controls. Arrows in J indicate ectopic projections and lines indicate expansion in the rostro-caudal dimension at the midline. The same ectopic projections were observed in the Robo2 knockout mice (M, dye injections, horizontal sections) compared to Robo1 knockout mice (K) and controls (L) at E18. Scale bar in C=250  $\mu$ m in A–C; bar in D=250  $\mu$ m; bar in F=250  $\mu$ m in E and F; bar in G=250  $\mu$ m; bar in H=100  $\mu$ m and bar in J=100  $\mu$ m in I, J, bar in M=200  $\mu$ m for K, L and M. Orientation in I is for all panels (R, rostral, C, caudal, D, dorsal, V, ventral).

mutants and control littermates (measurements shown in Table 1). Robo2 knockouts (*n*=7) displayed an increase in both the rostrocaudal (dotted line, Fig. 8C) and medio-lateral dimensions (dashed line with arrows, Fig. 8C) at E18 compared to wildtypes (*n*=6, Fig. 8A and Table 1); Robo2 heterozygote animals (*n*=8) displayed an intermediate phenotype for the medio-lateral dimension (Robo2-/- vs. Robo2+/- p<0.01, *t*-test; Robo2+/- vs. Robo2+/+ p<0.05, *t*-test, Table 1). Measurements for the Robo2-/- medio-lateral length showed a significant difference between Robo2-/- vs. +/+ (p<0.0005, *t*-test). Robo1 knockouts (*n*=8) had an expanded chiasm in the rostro-caudal dimension (Fig. 8B) compared to wildtype littermates (*n*=8) at E18 (p<0.01, *t*-test) but not compared to heterozygotes (*n*=6). The medio-lateral width however was unaffected.

In sagittal sections, defects in the RGC trajectory at the chiasm and ventral diencephalon were even more evident. In wildtype mice, RGC axons formed a tightly fasciculated bundle at the ventral diencephalon with, at most, one or two fibers leaving the main bundle (Figs. 3A and B). In Robo1–/– mice (n=6), we observed ectopic projections in the ventral diencephalon close to the normal optic chiasm (Figs. 3C, D). In Robo2–/– mice (n=6), we observed multiple bundles of ectopically projecting axons (Figs. 3E and F; arrows in F) as well as some axons projecting caudally toward the corticospinal tract (Fig. 3J; n=5; we did not trace these axons beyond the level of the pons however). In more dorsal regions, such as the telencephalic/diencephalic region of the internal capsule, we observed multiple bundles of axons (Fig. 3I) and an extensive amount of branching into the diencephalon (Fig. 3G).

The RGC axons from both Robo1 (Fig. 3K) and Robo2 (Fig. 3M) knockout mice reached their final target of the superior colliculus, indicating that some RGC axons are not affected by loss of Robo/Slit function, a similar result has recently been observed in Slit1/Slit2 double knockout mice (Thompson et al., 2006a).

## RGC axons deviate from the optic tract to enter the internal capsule and project exuberantly into the dorsal diencephalon

At the level of the internal capsule, axons defasciculated in the Robo2–/– mice and projected ectopically into the telencephalon either laterally toward the cortex or ventrolaterally toward the amygdala (Figs. 4E, G, I, and K). As the axons reached the level of the LGN in Robo2–/– mice, an abnormally large number of projections grew into the diencephalon compared to wildtype litter mates (Figs. 4H–K and 5D–G, n=8). Abnormal projections were evident on both the contralateral and ipsilateral sides of the diencephalon with respect to the single eye that was injected with DiI (compare Figs. 5D and G with F and I). A milder phenotype was observed in Rob2–/+ mice (Figs. 5E and E'; n=6). Abnormal projections were also observed in the Robo1–/– mice (Figs. 5H and H', compared to

controls in Figs. 5I and I'). Such optic tract abnormalities were recently reported in the Slit1/Slit2 double knockout mice (Thompson et al., 2006a).

## RGC axons project into the neocortex in Robo1 and Robo2 knockout mice

As described above, in Robo2-/- mice we observed RGC axons ectopically leaving the optic tract to enter the telencephalon. We observed several axon bundles per brain projecting into the cortex that apparently grew into random sites as there was no consistency between animals in terms of where these ectopic projections were observed in the rostro-caudal axis of the cortex (Figs. 6M–R, n=10). Axons grew from the intermediate zone to the pial surface, and formed what resembled terminal arborizations in the marginal zone and pial surface of the cortex (Fig. 6R. Ectopic projections into the cortex were also observed in Robo1-/mice, but the phenotype was much milder in that fewer ectopic projections were observed (Figs. 6G–L, n=6) and only 50% of the Robo1-/- had ectopic projections to the neocortex. Even in some wildtype mice we observed one or two stray axons within the cortex (Figs. 5A-F) indicating that these projections sometimes do occur normally, but are presumably corrected later. This may correspond to what has been observed by Chien and Hutson (Hutson and Chien, 2002) in the zebrafish where RGC axons make some aberrant projections into the brain even in the normal fish, which are later corrected.

In order to confirm that the RGC axons projected aberrantly to the neocortex of Robo knockout animals, we performed retrograde labelling placing DiI in the neocortex and looking for projections into the optic chiasm. Retrograde labelling from the cortex also revealed these ectopic projections (Fig. 7) from the retina as labelled axons were observed in the ventral diencephalon and optic chiasm for the Robo2–/– (Figs. 7E–H, n=3) and the Robo1–/– (Figs. 7I–L, n=3) compared to control animals (Figs. 7A–D, n=6).

## Discussion

The guidance of RGC axons from the retina to their targets is essential for the establishment of a functioning visual system. In this study, we provide evidence that the mammalian Robo proteins are potent regulators of retinal axon guidance *in vivo* and are critical for the correct targeting of RGC axons at multiple sites along their normal trajectory. Robo2 deficient mice developed severe axon guidance defects in the visual pathway, including the formation of ectopic projections into the contralateral optic nerve, additional axon bundles at the optic chiasm, axon growth defects at the level of the internal capsule resulting in ectopic projections into

Table 1

Rostro-caudal and medio-lateral length of the optic chiasm in Robo knockout mice

Genotype	Rostro-caudal length (±s.d.)	Medio-lateral length (±s.d.)
Robo1+/+ $(n=8)$	128 μm±4 μm	224 μm±9 μm
Robo1 $-/+$ ( $n=6$ )	136 $\mu$ m $\pm$ 6 $\mu$ m $p$ >0.1 vs Robo1+/+	223 $\mu$ m±15 $\mu$ m $p$ >0.1 vs Robo1+/+
	p > 0.1 vs  Robo 1 - / -	p>0.05 vs Robo1-/-
Robo1-/- (n=8)	$155 \ \mu m \pm 10 \ \mu m \ p < 0.01 \ vs \ Robo1+/+$	228 $\mu$ m $\pm$ 5 $\mu$ m $p$ >0.1 vs Robo1+/+
Robo2+/+(n=6)	134 μm±5 μm	240 μm±15 μm
Robo2-/+ (n=8)	145 $\mu$ m $\pm$ 6 $\mu$ m $p$ >0.1 vs Robo2+/+	268 $\mu$ m $\pm$ 12 $\mu$ m $p$ <0.05 $vs$ Robo2+/+
	p<0.001 vs Robo2-/-	p<0.01 vs Robo2-/-
Robo2-/- ( <i>n</i> =7)	175 $\mu$ m $\pm$ 7 $\mu$ m $p$ < 0.005 vs Robo2+/+	$301 \ \mu m \pm 5 \ \mu m \ p < 0.0005 \ vs \ Robo2+/+$



Fig. 3. Ectopic projections at the chiasm and in the ventral diencephalon of Robo1 and Robo2 knockout mice. One eye was injected with DiI (red labelling in all panels) and brains from wildtype (A, B, L), Robo1–/– (C, D, K) and Robo2–/– (E–J, M) mice were sectioned sagittally and counterstained with DAPI (blue labelling in all panels). In wildtype mice, axons within the chiasm form a single tight fascicle at the ventral midline (A, B; B is a higher power view of the white box in A) with only very few axons deviating from the main bundle (arrowhead in B). Ectopic bundles of axons at the chiasm are present in both Robo1 (C, D; D is a higher power view of the boxed region in C) and Robo2 knockout mice (E, F; F is a higher power view of the lower half of panel E; arrowhead in F depicts the original chiasm and arrows depict the ectopic projections). Retinal ganglion cell axons also projected ectopically caudally, apparently into the corticospinal tract (H and J; J is a higher power view of the region depicted by an arrowhead in H). In more dorsal regions of the diencephalon distinct ectopic bundles were observed (I; I is a higher power view of the boxed region in H) as well as an abnormally large amount of outgrowth into the dorsal diencephalon (G; G is a higher power view of the region depicted by an arrow in E). RCG axonal projection was normal in the superior colliculus in Robo1 (asterisk in K), wildtype (asterisk in L) and Robo2 knockout brains (asterisk in M). Scale bar in B=100  $\mu$ m; bar in C=500  $\mu$ m in A, C, E, H, K, L M and 200  $\mu$ m in D, bar in G=200  $\mu$ m; bar in J=200  $\mu$ m in F, J and I. Orientation in M is for all panels (R, rostral, C, caudal, D, dorsal, V, ventral).

the neocortex, and exuberant growth into the dorsal diencephalon (Fig. 8). Robo1 mice displayed similar, but far less severe defects in visual system projections indicating that Robo2 is the predominant Robo receptor in the development of the visual system.

## Expression of Robos and Slits in the optic pathway

By using Robo1 and 2 specific antibodies, we were able to determine the protein expression of these molecules in the visual system. Robo2 was expressed prior to Robo1 in RGCs, as previously described by *in situ* hybridization (Erskine et al., 2000; Ringstedt et al., 2000), and at higher levels at the optic chiasm. These earlier studies predicted, based on the relative expression patterns of Robo1 and Robo2 mRNA that Robo2 would be the predominant Robo receptor in the development of the visual system, and this is confirmed by our results given that the phenotype is more severe in Robo2 compared to Robo1 mutants. The expression analysis also suggests that the Robos act cell autonomously as in zebrafish (Fricke et al., 2001).



Fig. 4. RGC axons project ectopically into the internal capsule and within the diencephalon in Robo2 knockout mice. E17 coronal sections (A, D, E) and horizontal sections (B, C, F, G) of Robo2+/+ (A–D) and Robo2-/- (E–G) mice labelled with 68 kD neurofilament or 3A10 (neurofilament-associated protein) antibodies. In wildtype mice, RGC axons grow over the surface of the diencephalon from ventral to dorsal (A–D; C and D are higher power views of the boxed regions in B and A respectively). In Robo2-/- mice RGC axons deviate away from the optic projection and enter the internal capsule (E–G; G is a higher power view of the boxed region in F). When DiI (red labelling in H–K, coronal sections counterstained with DAPI (blue labelling)) is placed in the thalamus, both the thalamic projections to the cortex are labelled anterogradely, and RGC projections are labelled retrogradely from axonal branches into the dorsal thalamus in wildtype animals (H and J). In Robo2-/- mice (I and K), ectopic projections were observed in ventral regions in more rostral sections (H and I are matched sections, arrow in I depicts ectopic projection). In more caudal sections (J, K), the internal capsule is present and ventrally directed ectopic projections are also visible (arrow in K). Scale bar in E=400 µm in A, B, and F and 100 µm in D, E; bar in G=100 µm in C and G; bar in K=400 µm in H–K. Orientation in A is for panels A, D, E, H–K, orientation in B is for panels B, C, F and G (R, rostral, C, caudal, D, dorsal, V, ventral).

At the optic chiasm, Slit2 is expressed dorsal and caudal to the axons of the chiasm and Slit1 is expressed around the optic stalk and directly caudal to the axons in ventral regions of the tract (Erskine et al., 2000). In Slit1/Slit2 double knockout mice, it was found that the chiasm was expanded rostrally and caudally and thus, it has been postulated that Slit1/Slit2 act to determine the position of the optic chiasm by a mechanism of surround repulsion (Plump et al., 2002). Here we found an identical phenotype in the Robo2 mutant where the chiasm was expanded with ectopic bundles forming both rostrally and caudally. Therefore, at the optic chiasm, Slit acting through Robo is essential for the correct location of the chiasm and to keep the axons from decussating at ectopic locations.

# RGC axons from Robo knockout mice enter the contralateral optic nerve

We also observed the inappropriate extension of RGC axons into the contralateral optic nerve. This phenotype has also been observed in Slit1/Slit2 double knockout mice (Plump et al., 2002), EXT-1 conditional knockout mice, and EXT-1 heterozygous mutants crossed to Slit2 knockout animals (Inatani et al., 2003) and similar phenotypes have been observed in ext2 and ext13 mutant zebrafish (Lee et al., 2004). EXT-1 is an enzyme involved in the synthesis of heparan sulphate (HS) molecules and thus, this and other data demonstrating the binding of Slit2 to HS's (Hu, 2001; Liang et al., 1999; Ronca et al., 2001; Zhang et al., 2004), suggest that HS's are essential for Slit/Robo signalling. Collectively the data suggest that loss of Slit, Robo or HS's causes mistargeting of the RGC axons at the optic nerve.

## *RGC* axons leave the optic tract at the diencephalon to enter the internal capsule and project into the neocortex

Our analysis of Robo1 and 2 knockout mice showed the presence of substantial numbers of RGC axons entering the cerebral cortex. Surprisingly, even in some wildtype animals, approximately 1 axon fascicle per brain was found to mis-project into the cortex. Both of these phenomena were noted in Zebrafish wildtype and astray mutants (Hutson and Chien, 2002), where RGC axons turn aber-



Fig. 5. Ectopic projection of RGC axons project within the diencephalon in Robo1 and Robo2 knockout mice. RCG axon trajectory can be labeled by injecting DiI and DiA in the eye (schematic in panel A for the injection, panel B for the trajectory, and panel C to show the horizontal sections for D–I'). After growing over the surface of the diencephalon, RGC axons branch into the dorsal diencephalon (panel C, black arrow). This projection is small and restricted to the thalamus on the contralateral side of the eye injection (at E16 in wildtype brains, F and F' higher power image of F). In E16 Robo2+/– (E and E') and Robo2-/– (D and D') mice, this projection is much larger and is visible on both the ipsilateral and contralateral sides of the thalamus, even though only one eye was injected with DiI (D' and E' are higher power views of D and E respectively). Those aberrant projections were also observed at later stages (E18) in the Robo2-/– (G and G' high power of view). Robo1-/– also display a larger projection (H and H') compared to wildtype brains (I and I'). Scale bar in F'=200  $\mu$ m in D'–F' and 500  $\mu$ m in D–F; bar in I=500  $\mu$ m for G–I; bar in I'=200  $\mu$ m for G'–I'. OC, optic chiasm; white arrow in D–F and G–I shows the RCG projection (compared to the black arrow in schematic in panel C); dotted line in D–F and G–I delimited the midline. Orientation in C is for all panels (R, rostral, C, caudal, D, dorsal, V, ventral).

rantly to grow into the telencephalon. In zebrafish these aberrant pathfinding errors are later corrected in wildtype fish, but not astray mutants (note that zebrafish have only one Robo gene (astray) important for visual system development whereas Robol and Robo2 are important in mouse). Our results confirm that a similar phenomenon occurs in Robo knockout mice, but in this case RGC axons grow over long distances in the wrong direction and, upon reaching the cortex, have arborized axon endings in the superficial layers of the cortex. Interestingly Slit/Robo interactions are involved in axonal targeting in the olfactory system (Miyasaka et al., 2005) and recent experiments show increased arborization of RGC axons in zebrafish Robo2/astray mutants (Campbell et al., 2007). The

targeting errors we observed may be later corrected, or the misprojecting cells lost, but at present it is impossible to analyse this as the Robo mice die at birth.

Why do these axons make this incorrect pathfinding decision? In Robo2 knockout mice, RGC axons remained in a tightly fasciculated bundle as they grew over the hypothalamus, as they would in wildtype animals, until they reached the level of the internal capsule. At this point they deviated from the optic tract and turned laterally toward the cerebral cortex. Neither Slit1, 2 or 3 is normally expressed in the internal capsule (Marillat et al., 2002), thus this misprojection is not likely to be due to a Slit–Robo interaction, but this cannot be ruled out. This leaves three alternatives for the mis-



Fig. 6. Robo1 and Robo2 knockout mice display aberrant RGC projections into the neocortex. Lipophillic dye tracing of RGC axons in E18 wildtype (A–F), Robo1–/– (G–L) or Robo2–/– (M–R) mice. DiI injections (in red) were performed unilaterally into the right retina (A–R). After diffusion of dye, brains were dissected and sections cut sagittally and counterstained with DAPI (in blue), and viewed by confocal microscopy. In wildtype mice, very few axons are present in the cortex from an injection of DiI into the eye (A–F); three different examples from 3 different brains are shown, B, D and F are higher power views of the region shown by an arrow in A, C and E respectively; arrows in B and F show aberrant axons in the cortex of wildtype animals). In Robo1–/– mice (G–L), and even more so in Robo2–/– mice (M–R), the ectopic projections into the cortex are vastly increased (G–L; three different examples are shown, H, J and L are higher power views of the region shown by an arrow in G, I and K respectively). In Robo2–/– mice, ectopic RGC projections grew to the marginal zone (top arrow in R; three different examples are shown 1) M–O, 2) P and 3) Q, R); N and O are higher power views of the regions shown by an arrow in Q). Note the projections appear to grow up through the intermediate zone, enter the cortex and continue to grow up to layer one (arrows in R). These axons appear to terminate as highly branched axons that resemble termination zones in the superior colliculus (the normal target of RGC axons). Scale bar in B=50  $\mu$ m; bar in F=500  $\mu$ m in A, C, E, G, K, M, Q, 200  $\mu$ m in I, M, O, P, 100  $\mu$ m in D and 50  $\mu$ m in F; bar in H=20  $\mu$ m; bar in J=50  $\mu$ m; bar in L=50  $\mu$ m, bar in K=100  $\mu$ m. Orientation in M is for all panels (R, rostral, C, caudal, D, dorsal, V, ventral).

projection phenotype: 1) Robo interacts with another ligand at the internal capsule that usually repels the axons away from this region; 2) retinal axons are being misguided by another population of axons affected in the Robo2 mutant or 3) Robo2 homophilic or Robo1/Robo2 heterophilic interactions are responsible for keeping the axons within the optic tract after they reach the internal capsule. However, the latter argument is less likely, because it is at this point in the pathway (as the axons grow over the dorsal diencephalon) where the axons normally splay apart to branch within the dorsal diencephalon. Therefore, in normal animals, axon–axon interactions are less likely in this region of the pathway.

## RGC axons do not ectopically project into the hypothalamus but exuberantly project into the dorsal diencephalon in Robo2 knockout mice

It has been previously shown that the hypothalamus provides repulsive cues to RGC axons (Tuttle et al., 1998) and Slit expression has been correlated with this repellent activity (Ringstedt et al., 2000). As RGC axons grow over this region of the hypothalamus they remain tightly fasciculated, but then spread out once they reach the dorsal diencephalon. The dorsal lateral region of the diencephalon (LGN) receives inputs by way of branches from approximately one third of the RGC axons (Bhide and Frost, 1991). The LGN does not express any Slit family member (Marillat et al., 2002; Ringstedt et al., 2000) and instead expresses a growth promoting activity that is as yet unidentified (Tuttle et al., 1998). It has also been postulated that the lack of Slit2 in this region allows the axons to spread out and defasciculate over this region to promote branching. Our data suggest that Robo2 does play a role in the normal targeting of axon branches to the LGN. We observed a large increase in axons (possibly axonal branches) in the LGN of Robo2 knockout mice (and an intermediate phenotype in Robo2+/- mice). We also observed ectopic branching into the ventral diencephalon (hypothalamus) in the absence of Slit/Robo signalling at the level of the optic chiasm (Figs. 2D, G, H) as the previous model would have suggested. The RGC axons remained fasciculated as they grew over the hypothalamus until they reached the level of the internal capsule where they began to deviate from the main bundle of axons. In the LGN it is possible that the attractive activity previously described may be partially suppressed by Slits diffusing into this area from the surrounding regions of the diencephalon that express Slit mRNA (Marillat et al., 2002;



Fig. 7. Retrograde labelling from the cortex to the optic chiasm in Robo1 and Robo2 knockout mice. DiI was injected into the cortex of E18 wildtype mice (A–D, sagittal sections counterstained with DAPI) retrogradely label cells within the thalamus, but do not normally label cells in the optic tract. In Robo2–/– mice (E–H, sagittal sections) retrogradely labelled projections are visible from the cortex, through the internal capsule to the ventral forebrain and into the optic chiasm (arrows in G and H). In Robo1–/– mice (I–L, sagittal sections) retrogradely labelled projections are visible from the cortex, through the internal capsule to the ventral forebrain (arrows in K and L) Scale bar in H=250  $\mu$ m for panels A–H, bar in L=500  $\mu$ m for panels I–L. Orientation in D is for all panels (R, rostral, C, caudal, D, dorsal, V, ventral).

Ringstedt et al., 2000). This inhibition may not be present once this area is dissected out for *in vitro* analysis. Another possible explanation is that there is an additional molecule involved that normally represses growth and prevents the axonal branching from the two thirds of RGC axons that normally do not innervate this region, and in the knockout this signalling is eliminated, thus causing more axons to branch into the dorsal diencephalon. Robo2 itself is expressed in the LGN early in development (Marillat et al., 2002) and can bind homophilically (Liu et al., 2004), and thus could act to repel branches from Robo2 expressing axons, however no direct evidence for this possibility currently exists.

### Conclusions

Robo2 is required for the correct targeting of RGC axons from the retina to the superior colliculus. Our data suggest several instances where Robo likely interacts with Slit to mediate these phenotypes, such as at the optic nerve and optic chiasm, but also places where Robo may be interacting with another ligand or homophilically with itself, such as at the level of the internal capsule and in the dorsal diencephalon. Together, these results present new insights into the normal pathfinding of retinal axons post-chiasm, namely that some (very few) axons can stray into the cortex even in some wildtype animals, and provide evidence for additional molecules involved in Robo mediated axon guidance in the visual pathway. Finally, our data do not support a model whereby Slit, expressed by the hypothalamus, keeps Robo expressing axons from entering this region. Future experiments will include studies of transheterozygotes and possibly double Robo1 and Robo2 knockouts in this system.

### **Experimental methods**

### Animals and mouse genotyping

Animals were generated in either the animal house facility of Kings College, London, UK, University College, London, UK or The University of Queensland, St Lucia, Australia, under the approval of the ethics committee for each Institution and the Home Office (in the case of the Institutions in the UK). Experiments were performed at Kings College London, University College, London or The University of Queensland. Some of the fixed brains were shipped to The University of Maryland for experimental analysis. The generation of Robo1 and Robo2 deficient mice have been previously described (Andrews et al., 2006; Lu et al., 2007). For genotyping of Robo1 mice carrying the full transgene, we used Polymerase Chain Reaction (PCR) primers Robo1KO F (5'-CGAGGARGAAARSTSATGATC-3') and Robo1KO R (CCACAAGACTTGTGACAATACC). For genotyping of Robo2 knockout mice, two separate PCRs were carried out, the first to amplify across the deleted region EKOF2 (5'-ATTTGCTGAGAGCAGGCATT-3') and MEBAC13R (5'-AAATGAAATATCCCCAAATTAGAGC-3'), and the second to amplify across exon5 MEBACF16 (5'-TCTTTTTCTGCTTTGAACAACAA-3') and MEBAC13R. After an initial 5-minute denaturation at 94 °C, 30 cycles of 94 °C for 30 s, 55 °C for 1 min, and 72 °C for 2 min were performed, followed by a final 10-minute extension at 72 °C. Twenty microliters of the PCR product was analysed by electrophoresis on a 2% agarose gel, and bands visualised under UV illumination following ethidium bromide staining.

### In situ hybridization

*In situ* hybridization experiments were performed on mouse embryo (E15.5) specimens, using a modified protocol based on Wilkinson (1992) and Henrique et al. (1995), as described by Camurri et al. (2004). Sense and antisense digoxigenin (DIG) riboprobes were synthesised by incorporation of



Fig. 8. A summary of the aberrant RGC projections in Robo1 and 2 knockout mice. RCG projections are represented in ventral views (A–C) to demonstrate the optic nerve and chiasm and sagittal views (D–F) to demonstrate the visual projection post-chiasm. In wildtype mice (A) and Robo1 knockout mice (B), the majority of the RCG axons project contralaterally at the optic chiasm (OC) whereas in Robo2 knockouts (C) a large number of axons cross the midline but also project into the contralateral optic nerve and display an ectopic caudal extension (red axons at the midline). Both Robo1 (B) and Robo2 knockouts (C) displayed an expansion of the chiasm in the rostro-caudal dimension (dotted line) but only Robo2 mutants showed a medio-lateral expansion (dashed line). In wildtype mice, after leaving the chiasm, RGC axons then project over the diencephalon from ventral to dorsal, branch into the dorsal diencephalon and then project back to the superior colliculus (SC; D). In Robo1 (E) and Robo2 (F) knockout mice, axons form ectopic bundles in the region of the OC, and then take one of three projection routes: 1) the normal projection over the diencephalon to the SC, but display an increased projection into the dorsal diencephalon; 2) the axons project ectopically into the internal capsule and into the neocortex forming termination zone-like structures (50% of the cases for Robo1 knockout, dotted line projection); and 3) they project ectopically down the corticospinal tract (Robo2 knockout only), possibly fasciculating with CST axons that express both Robo1 and Robo2 through homophilic binding. These data suggest that Robo2, and to a lesser extent Robo1, are essential for the proper pathfinding of RGC axons at key decision points; the OC, and the ventral and dorsal regions of the diencephalon. OC, optic chiasm; SC, superior colliculus, CST, corticospinal tract. Orientation in A is for panels A, B and C; orientation in D is for panels D, E and F (R, rostral, C, caudal, D, dorsal, V, ventral).

DIG-labelled UTP (Boehringer Manheim) from linearised Robo2 template using T3 and T7 RNA polymerase (Promega). Embryos were embedded in OCT before cryo-sectioning.

#### Immunohistochemistry

Brains were collected between E12.5 and E18.5. Embryos were either immersion fixed directly in 4% paraformaldehyde or transcardially perfused with saline, followed by 4% paraformaldehyde. They were then postfixed in the same fixative solution overnight, and transferred to phosphate-buffered saline (PBS; pH 7.4) until sectioning and immunohistochemistry, performed as previously described (Andrews et al., 2006). Brains were blocked in 3% agar and cut at 40  $\mu$ m on a Vibratome (Leica, Deerfield, IL) or 30  $\mu$ m on a cryostat. Primary antibodies used were mouse monoclonal antibody against neurofilament-associated protein (3A10), obtained from the Developmental Studies Hybridoma Bank or a rabbit anti-68kD neurofilament antibody (Chemicon) both used to demonstrate the presence of axons (Phelps et al., 1999; Serafini et al., 1996), or rabbit anti-Robo1 or rabbit anti-Robo2 (1:10,000 and 1:5000 respectively; (Andrews et al., 2006; Sabatier et al., 2004). Secondary antibodies used were biotinylated rabbit anti-mouse secondary antibody (1:500; Vector Laboratories) for 2 h or biotinylated donkey anti-rabbit (1:500; Vector Laboratories). The signal was amplified using an avidin-biotin kit (ABC kit; Vector Laboratories) and then sections were immersed in nickel-3,3'-Diaminobenzidine (DAB) chromogen solution (2.5% nickel sulfate and 0.02% DAB in 0.175 M sodium acetate) activated with 0.01% (v/v) hydrogen peroxide until formation of a purple to black coloured precipitate. The reaction was stopped with 1× PBS; sections were then washed several more times in 1× PBS, then mounted on gelatin-coated slides (0.5% gelatin) and dried overnight before coverslipping with D.P.X. mounting medium (Electron Microscopy Sciences, PA).

Labelling was analysed with a light microscope (Leica). Images were scanned with a Power Phase digital camera (Phase One, Coppenhagen, Denmark) directly into Adobe Photoshop software.

#### Carbocyanine dye tracing

Carbocyanine dyes were used in embryonic Robo wildtype (+/+), heterozygote (+/-), and mutant (-/-) brains between E16 and E18 to label

the visual projection and retrograde labelling of aberrant projections into the cerebral cortex using methods previously described, (Andrews et al., 2006). Other brains were labeled by placing a single crystal of either DiI or DiA in the brain as previously described (Molnar et al., 1998).

For complete optic nerve labelling, embryonic mouse tissue was fixed in 4% paraformaldehyde in 1× PBS, one or both eye enucleated, and DiI (and in some cases DiA) was injected unilaterally into the optic disk (and in some cases bilaterally). Heads were incubated at 37 °C in 1× PBS containing 0.2% sodium azide for one week. In order to visualize the optic chiasm, some brains were carefully dissected and the proximal visual system was imaged enface with a fluorescence microscope.

For all other dye tracing, labeled brains were stored at 37 °C in darkness for between 2 and 6 weeks to allow for dye transport before sectioning. Brains were then placed in 4% agarose blocks and cut at either 40  $\mu$ m, 80  $\mu$ m or 100  $\mu$ m using a Vibratome (Leica). Injection sites were verified after sectioning by the presence of a fluorescent bolus and a pipette track. The sections were washed and incubated overnight with 4'-6-Diamidino-2-Phenyllindole (DAPI, 1:20,000, Sigma) in 1× PBS or bisbenzimide (10 min in 2.5  $\mu$ g/ml solution in 1× PBS, Sigma). They were then rinsed, mounted and cover-slipped with poly vinyl alcohol (PVA)/DABCO mounting medium. Images were collected using a laser scanning confocal microscope (Fluoview FV5000 Olympus configured on an Olympus BX61 microscope, Melville, NY or Leica, Microsystems UK Ltd). Sequential images collected using the Leica microscope were subsequently reconstructed using Metamorph imaging software (Universal Imaging Corporation).

### Acknowledgments

VS was a recipient of an MRC (UK) Career Establishment Award. The work in JGP's laboratory was supported by a Wellcome Trust Grant (074549). The work in LJR's laboratory was supported by grant #FY05-119 from The March of Dimes Foundation for Birth Defects. LJR is a Senior Research Fellow supported by the National Health and Medical Research Council, Australia. We thank Prof. Fuji Murakami, Osaka University, Japan for generously providing the Robo1 and Robo2 specific antibodies.

### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mcn.2007.12.017.

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