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DISSERTATION

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ROBO-SWAPS SPECIFY THE FUNCTIONS OF THE ROBO RECEPTORS AS AXON GUIDANCE MOLECULES

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Zusammenfassung

Drei Robo-Rezeptoren wurden in der Fruchtfliege *Drosophila melanogaster* entdeckt und beschrieben Robo, Robo2 und Robo3 haben unterschiedliche und gemeinsame Funktionen im embryonalen zentralen Nervensystem (ZNS) und sie sind konserviert. Als repulsive axon-guidance Moleküle kontrollieren sie zwei sehr wichtige Prozesse. Indem sie ihren gemeinsamen Liganden Slit binden beeinflussen sie das Überqueren der Mittellinie und die laterale Positionsfindung des rechtwinklig angeordneten , symmetrischen Nervensystems. Die bis jetzt entdeckten Funktionen der Robo Rezeptoren können entweder ihren unterschiedlichen Proteindomänen oder ihren verschiedenen raumzeitlichen Expressionsmustern zugeschrieben werden. Die Proteindomänen der Robo Rezeptoren unterscheiden sich hauptsächlich intrazellulär. Während Robo vier konservierte Domänen hat, besitzen Robo2 und Robo3 nur zwei von diesen. Die Expressionsmuster sind für alle drei Robo Rezeptoren verschieden. Auch in den Expressionsmustern unterscheiden sich die drei Robo Rezeptoren. In den frühen Entwicklungs-Stadien sind Robo und Robo2 in allen Neuronen exprimiert, in späteren Stadien bilden alle drei Robo Rezeptoren unterschiedliche und überlappende Zonen. Robo wird immer von allen Neuronen über die gesamte Breite des ZNS exprimiert, Robo3 ist auf die lateralen zwei Drittel beschränkt und Robo2 wird nur noch im äussersten lateralen Drittel exprimiert.

In dieser Arbeit wollen wir die Frage klären, ob die unterschiedlichen Funktionen der Robo Rezeptoren die verschiedenen Proteindomänen oder die unterschiedlichen Expressionsmuster wiederspiegeln. Um dies zu beantworten manipulierten wir die genomischen Loci der Robo Rezeptoren mittels homologer Rekombination. Dadurch konnten wir die kodierende Region jedes Robo Gens mit der kodierenden Region jedes der anderen Robo Gene austauschen. Auf diese Weise erreichten wir die Produktion eines jeden der unterschiedlichen Robo Rezeptoren in den verschiedenen Expressionsmustern der endogenen Loci aller anderen Robo Rezeptoren und dies ermöglichte uns zu fragen: sind die Robo Rezeptoren funktionell untereinander austauschbar? Wenn ja, sind ihre Funktionen in den unterschiedlichen raumzeitlichen Expressionsmustern kodiert. Wenn nein sind die Funktionen in den Eigenschaften der unterschiedlichen Proteindomänen verschlüsselt. Wir können zeigen, dass Robo weder von Robo2 noch von Robo3 in seiner repulsiven Funktion an der Mittelline ersetzt werden kann. Dies bedeutet, dass Robo eine einzigartige Funktion hat, die in seinen Proteindomänen kodiert ist . Weiterhin können wir zeigen, dass *robo3* seine Funktion des lateralen Positionierens nur durch Genexpression kontrolliert, da Robo3 vollständig durch

Robo und Robo2 ersetzt werden kann. Dieses Ergebnis stimmt mit Modellen überein, die die Funktion des lateralen Positionierens durch die Gesamtmenge an allen Robo Rezeptoren auf einem Axon kodiert sehen und diese Funktion dadurch in allen drei Robo Rezeptoren gleichermassen vorhanden ist. Die Ergebnisse des robo2 locus deuten darauf hin, dass Robo2 eine diffizilere Rolle spielt. Wir zeigen, dass Robo2 bifunktional ist und nicht nur als repulsiver Rezeptor dient, sondern auch das Kreuzen von Neuronen fördern kann. Diese Funktion wird allerdings nur im NetrinAB doppelmutanten Hintergrund offenbar, wenn also keine weiteren attraktiven Kräfte an der Mittellinie vorhanden sind.

Summary

In the fruit-fly *Drosophila melanogaster*, three Robo receptor family members (Robo, Robo2 and Robo3) with distinct and overlapping functions in the embryonic CNS have been described. These receptors are conserved and control as repulsive axon guidance molecules two important processes. Via controlling midline crossing and lateral positioning, they shape the orthogonal array of the neuropile in response to their common ligand Slit. Distinct functions of the three known Robo receptors could be attributed to different features of the respective protein domains or to differences in their spatiotemporal expression patterns. Structurally, the Robo receptors differ mainly in their cytoplasmic regions, since Robo2 and Robo3 lack two conserved domains with respect to Robo. The expression patterns are distinct for all three Robo receptors. Robo and Robo2 are expressed ubiquitously in early stages but in later stages all three Robo receptors form distinct and overlapping regions of expression. Robo is expressed on the entire width of the embryonic CNS whereas Robo3 is restricted to the lateral two thirds and Robo2 is restricted to the outermost one-third of the CNS.

Here, we address whether the distinct axon guidance functions of the three Robo receptors reflect different features of the proteins or differences in their expression patterns. In order to answer this question we used homologous recombination to replace the coding region of each *robo* gene with each one of the other three *robo* genes. Thus, we are able to express a particular Robo receptor in the endogenous loci of all the other Robo receptors. Now, we can ask whether one particular Robo receptor is replaceable by the others. If yes, the functions are encoded in the different expression patterns of the genomic loci. If no, the functions lie within the distinct features of the protein domains. We show that *robo* can not be replaced by *robo2* or *robo3* in its midline repelling function. This reflects the structural uniqueness of the Robo receptor. However, *robo3* can be fully replaced by either *robo* or *robo2*. Thus, Robo3`s lateral positioning function appears to be structurally present in all three Robo receptors. These results are consistent with models in which total levels of Robo receptors specify lateral positions. The results for the Robo2 receptor suggested a more complex role for Robo2 in midline crossing than previously assumed. In further experiments we discovered that Robo2 is not only a repulsive receptor but has an unexpected positive contribution to midline crossing. This function is only revealed in a background devoid of any attraction mediated by the Netrin-molecules.

Introduction

Our nervous system is required to respond to most of the tasks life challenges us with. Therefore it is of prime importance that it develops correctly. In order to understand this development better and to get a chance on restoring defects, scientists investigate various model organisms with a multitude of techniques.

We chose as a model the central nervous system (CNS) of the *Drosophila melanogaster* embryo. The organism itself is easy to handle, provides many powerful genetic and analytical tools and gives us the possibility to manipulate easily the well conserved molecules and mechanisms. For axon guidance, the CNS of the fruit fly embryo is a handy model since it is - compared to vertebrates - of a simpler build-up. It is relatively flat, the neuronal positions are known and specific single axons can be visualized by several markers (Thomas, Bastiani et al. 1984).

Basic mechanisms in axon guidance

Neurons consist of a cell body, axons and dendrites. Initially, a differentiating neuron produces many protrusions - the neurites - but only one of them gets stabilized and forms the axon. This axon grows towards its target and - after having reached it - establishes synaptic connections. The target can possibly be several thousand cell-diameters away. To chop these long distances into shorter pieces, guidepost cells are located at strategic intervals providing the required information at so-called choice-points (Clagett-Dame 1998; Holtmaat, Oestreicher et al. 1998; Holtmaat, De Winter et al. 2002; Endo 2007). In order to direct the axons, the choice points can either provide permissive cues - therefore allowing growth - or instructive cues, providing directionality through attraction or repulsion. These instructive features of axon guidance cues can act at long and at short ranges, depending on their localisation. Secreted cues can diffuse away from their source and form gradients, restricted cues are attached to the membranes of their producing cells (Tessier-Lavigne and Goodman 1996).

Usually, pioneering neurons lead the way through a relatively axon-free environment, while followers merely follow the channelled paths. But at specific choice points the followers have to stop doing so and find their own defined pathway and individual synaptic partners. Axon-guidance molecules help the neurons to accomplish this task

with a specific combination of receptors recognizing the ligands in the surrounding environment (Thomas, Bastiani et al. 1984; Tessier-Lavigne and Goodman 1996).

Axon guidance molecules

It is difficult to put axon guidance molecules into discrete functional classes because most of the families comprise members with different, even opposite, features. For example, the Semaphorin-family contains both cell-attached and diffusible members indicating short- and long-range mechanisms respectively (Kolodkin, Matthes et al. 1993). Additionally, many guidance molecules are bi-functional, attracting some and repelling other axons. The distinct response of one axon is thought to depend on the specific set of receptors expressed on the tip of the axon, recognizing their cognate ligands (Nose, Takeichi et al. 1994; Colamarino and Tessier-Lavigne 1995). However, molecules and mechanisms in axon-guidance seem to be very well conserved. Discoveries in both insects and vertebrates contributed largely to our understanding of the developing nervous system (Goodman 1994). But it is still unclear how the complex interplay between the multitudes of interaction-partners is regulated.

Cell adhesion molecules (CAMs)

CAMs are transmembrane cell surface molecules which can function as both ligands and receptors. They are adhesion molecules and signal transducers simultaneously (Gottardi and Gumbiner 2001). Their signalling responses include enhanced calcium influx through G-protein dependent channel activation, pH change and phosphoinositide turnover (Maness and Schachner 2007). Mostly they convey their function through homophilic, in rarer cases through heterophilic interactions (Kuhn, Stoeckli et al. 1991; Kovalick, Schreiber et al. 1998). Two main families are known to be involved in axon-guidance. One is the immunoglobulin- (Ig) superfamily with the L1-type family as one of its most famous members in vertebrates (Castellani, De Angelis et al. 2002) and its only L1-type family-member Neuroglian (Nrg) in *Drosophila* (Hortsch 2000). The other main family comprises the cadherin-superfamilies (Hu and Rutishauser 1996) including neuronal CAM (N-CAM) in vertebrates (Cremer, Lange et al. 1994) and its homolog FasciclinII (FasII) in *Drosophila* (Lin, Fetter et al. 1994). But also other families like the Leucine-rich (LRR) repeat (Krantz and Zipursky 1990) and FasciclinI (FasI) families (Elkins, Hortsch et al. 1990) have been reported.

Extracellular matrix molecules (ECMs)

Many ECM molecules seem to be involved in axon-guidance through promoting or inhibiting axon outgrowth. Such families include the laminin, the tenascin, the collagen and the thrombospondin families as well as fibronectins and proteoglycans (Bixby and Harris 1991; Hynes and Lander 1992; Schachner, Taylor et al. 1994).
Proteoglycans for example were first discovered to be important in fibroblast-growth-factor- (FGF) signalling (Olivier, Raabe et al. 1993; Spivak-Kroizman, Lemmon et al. 1994) before they have been reported to promote neuronal migration, axon guidance and synapse formation (Rhiner and Hengartner 2006). The assembly of the proteoglycans could explain their functions in increasing the complexity in neural wiring. They have core proteins (in *Drosophila*: one syndecan and two glypicans) with linear polysaccharides attached and belong to the glycosaminoglycan family (GAG) of macromolecules (Kjellen and Lindahl 1991). These chains of disaccharides get heavily and very diversely modified, in particular by sulfates, generating numerous protein binding sites and regulatory properties (Turnbull, Powell et al. 2001).

Receptor protein tyrosine kinases (RTKs)

Different RTKs have been identified which influence axon growth, target invasion and also axonal branching (Barbacid 1995). These RTKs include the receptors for fibroblast growth factors (FGFRs) and the Trk family of neurotrophin receptors (Basilico and Moscatelli 1992; Barbacid 1995).

The largest subfamily of RTKs in vertebrates is the Eph-family and consists of the Eph receptors and their membrane bound ligands, the ephrins (Cheng, Nakamoto et al. 1995; Drescher, Kremoser et al. 1995). The ephrins and Eph-receptors comprise two classes, depending on which anchor keeps them at the membrane: ephrin-As are anchored through a phospholipid, the glycosylphosphatidylinositol (GPI) and bind to the EphA receptors. As a second class, EphB receptors recognize ephrin-Bs which have a transmembrane domain (van der Geer, Hunter et al. 1994). Ephrins and Eph-receptors have many roles, amongst others they are important in the formation of topographic maps and for axon fasciculation (Cheng, Nakamoto et al. 1995; Drescher, Kremoser et al. 1995; Winslow, Moran et al. 1995; Zhang, Cerretti et al. 1996). They can act as contact repellents and as attractants and they can signal in two directions, serving either as a ligand or as a receptor (Hindges, McLaughlin et al. 2002; Mann, Ray et al. 2002).

In *Drosophila*, a well known RTK is Derailed (Drl) which plays a role in regulating axon fasciculation (Callahan, Muralidhar et al. 1995) and in the selection of one of the two commissures - the anterior or the posterior commissure (Yoshikawa, McKinnon et al. 2003) (see below in: morphogens).

Morphogens

Morphogens are signalling molecules which are expressed and secreted from a certain region and build a concentration gradient emanating from their source. Cells within this gradient differentiate according to their position and therefore distance to the morphogen-source (Mehlen, Mille et al. 2005).

Wnts are one class of morphogens important in axon guidance. Originally, they were identified for their roles in cell proliferation and cell-fate specification. Now, it has been shown that they have additional, bifuntional tasks in axon guidance, attracting and repelling axons over long distances (Lyuksyutova, Lu et al. 2003; Yoshikawa, McKinnon et al. 2003; Liu, Shi et al. 2005). One of the still discussed models proposes that the outcome of Wnt-signalling depends on whether the intracellular signalling complex includes the seven-pass transmembrane receptor Frizzled (Fz) or a receptor of the Related to tyrosine kinase (Ryk) family (Imondi and Thomas 2003). Fz-receptors could be linked through their “planar-cell-polarity-pathway” and/or “calcium-pathway” to the cytoskeleton and mediate attraction (Zou 2004). However, it is unknown how Ryk-receptors would communicate their repulsive function since no signalling pathways have yet been discovered (Yoshikawa, McKinnon et al. 2003; Liu, Shi et al. 2005).

In mice it is the morphogen Sonic hedgehog (Shh) which seems to mediate attraction in the dorso-ventral system of the embryonic CNS. It attracts commissural neurons towards their temporary target - the floorplate – through Smoothened (Smo) (Charron, Stein et al. 2003). Smo is connected to Shh by cell-adhesion-molecule-related/down regulated by oncogenes (Cdon) and biregional Cdon-binding protein (Boc) (Okada, Charron et al. 2006). But morphogens can also convey repulsion as shown by bone morphogenetic proteins (BMPs). In particular, BMP7 repels commissural neurons from the mammalian roofplate (Augsburger, Schuchardt et al. 1999; Gherardi, Youles et al. 2003).

In *Drosophila,* Wnt5 seems to control the decision of which of the two commissures present in the embryonic CNS – anterior or posterior - to choose. The decision is made

by the repulsive Wnt5-receptor derailed (Drl). Drl is expressed by neurons of the anterior commissure which avoid the diffusible ligand Wnt5, spreading from neurons close to the posterior commissure (Figure 1) (Yoshikawa, McKinnon et al. 2003).

But Wnts, Shh and BMPs are not the only morphogens involved in axon guidance (Jessell, Bovolenta et al. 1989). The FGF- receptor was found to influence neurite outgrowth (Doherty and Walsh 1996). Examples are FGF2 in the *Xenopus* visual system (McFarlane, McNeill et al. 1995; McFarlane, Cornel et al. 1996; Webber, Hyakutake et al. 2003) and FGF8 as an attractant for trochlear motor axons in the vertebrate hindbrain (Irving, Malhas et al. 2002).

Semaphorins and their receptors

Semaphorins are a large family of cell-surface and secreted proteins which are defined through a Semaphorin domain at their N-terminus (Kolodkin, Matthes et al. 1993). Vertebrates have 20 family members and *Drosophila* has only five members (C.S Goodman 1999). The families are divided into eight classes and signal through mulitmeric receptor complexes. Most of these complexes include a member of the plexin-family (Winberg, Noordermeer et al. 1998; Winberg, Tamagnone et al. 2001). Other receptors included in the signalling-complexes are the neuropilins for the class 3 semaphorins and Integrins for the class 7 semaphorins (Yazdani and Terman 2006). The main function of the Semaphorins seems to be an inhibitory one, acting over short-ranges (Tamagnone, Artigiani et al. 1999; Raper 2000). In order to do this, they induce growth-cone collapse by signalling to the actin cytoskeleton (Kolodkin, Matthes et al. 1993; Luo, Raible et al. 1993) but also attractive roles for some axons have been reported (Raper 2000).

Netrins and their receptors

The Netrins are a small family of well conserved, bifunctional guicance cues (Kennedy, Serafini et al. 1994; Serafini, Kennedy et al. 1994; Culotti and Kolodkin 1996; Harris, Sabatelli et al. 1996; Mitchell, Doyle et al. 1996). They consist of a stretch of Laminin N-terminal- (LamNT) domains, three epidermal growth factor- (EGF) like repeats and a C-terminal domain which is enriched in the RGD motif, a known recognition sequence for members of the integrin family (Figure 2) (Harris, Sabatelli et al. 1996; Mitchell, Doyle et al. 1996). Netrins are expressed and secreted from the midline and their receptors belong to the Ig-superfamily, mediating either attractive or repulsive responses, depending on

the receptor-type which binds. The receptors can belong to either the deleted in colorectal cancer- (DCC) family which conveys attraction or to the uncoordinated5- (Unc5) family for repulsive functions (Kolodziej, Timpe et al. 1996; Keleman and Dickson 2001).

In flies, the DCC-family member is Frazzled (Fra) and it mediates mostly an attractive response in CNS-neurons. It is – like the vertebrate DCC - a single-pass transmembrane receptor, contains four Ig-domains, six fibronectin III- (FNIII) repeats and a cytoplasmic domain with three conserved regions: P1 to P3 (Figure 2). Its expression is uniformly on all neurons at all stages (Kolodziej, Timpe et al. 1996; Hong, Hinck et al. 1999). However, Fra seems to be involved in repulsive axon guidance as well (Kolodziej 1997; Keleman and Dickson 2001). The member of the other family – the Unc5-family – is a single-pass transmembrane receptor which can mediate repulsive responses in a subset of motor axons that exit the *Drosophila* CNS without crossing the midline, avoiding muscles which express Netrins (Keleman and Dickson 2001). *Drosophila* has two redundant Netrin-ligands: NetrinA and NetrinB (NetA, NetB) (Harris, Sabatelli et al. 1996; Mitchell, Doyle et al. 1996).

Axon-guidance through Netrins seems to be well conserved since mammals and worms use Netrins and their receptors as well. The vertebrate homologs are Netrin-1 and Netrin-2, the Fra homolog is called DCC/Neogenin. In worms the receptor is called UNC40 and it responds to the ligand UNC6. Repulsive receptors are the *C.elegans* UNC5 and the vertebrate homologs Unc5a- Unc5d (Kennedy 2000; Huber, Kolodkin et al. 2003; Garbe and Bashaw 2007; Round and Stein 2007).

Slit and its receptors

Slit has been identified in *Drosophila* and it is a large repulsive ligand for the roundabout- (Robo) receptor family (Seeger, Tear et al. 1993; Kidd, Brose et al. 1998; Kidd, Bland et al. 1999). Slit consists of four (LRRs), seven EGF-like domains, a laminin G domain and a cystein knot at the C-terminal end (Figure 3) (Rothberg, Jacobs et al. 1990). The EGF region is subject to cleavage by an unkown protease (Brose, Bland et al. 1999) and gives rise to two proteolytic fragments with distinct activities, an N-terminal fragment with repulsive activity and an inactive C-terminal fragment (Wang, Brose et al. 1999; Nguyen Ba-Chervet, Brose et al. 2001).

There is only one *slit*-gene in *Drosophila,* but three Slits have been identified in mammals (Slit-1, Slit-2 and Slit-3) with redundant functions (Itho, Miyabayashi et al. 1998; Brose, Bland et al. 1999; Li, Chen et al. 1999).

The repulsive function of Slit is transduced through the single-pass transmembrane receptors of the Robo-family (Figure 3) which bind Slit within a similar range (Simpson, Bland et al. 2000[a;] Howitt, Clout et al. 2004). Extracellularly, they consist of five Ig-domains and three FNIII- domains (Kidd, Brose et al. 1998). Intracellularly, they have no reported catalytic activity, but they bear four conserved consensus motifs (cc0, cc1, cc2 and cc3) with different cytoplasmic signalling proteins as interaction partners (see below) (Bashaw, Kidd et al. 2000; Wong, Ren et al. 2001; Fan, Labrador et al. 2003; Lundstrom, Gallio et al. 2004; Hu, Li et al. 2005).

The three fly Robo receptors form a particular expression pattern, referred to as the Robo-code (see below) (Rajagopalan, Vivancos et al. 2000[a;] Simpson, Bland et al. 2000[a]). But the Robo receptors do not only differ in their particular expression but also in their intracellular composition. Whereas Robo bears all four cc-domains, Robo2 and Robo3 appear to have only cc2 and cc3 (Figure 3) (Rajagopalan, Vivancos et al. 2000[a;] Simpson, Bland et al. 2000[a]).

Like Slit, Robos are highly conserved and in mammals there are four Roundabouts: Robo1, Robo2, Robo3 (also known as Rig1) and Robo4 (better known as magic Roundabout) (Kidd, Brose et al. 1998; Brose, Bland et al. 1999; Yuan, Cox et al. 1999; Huminiecki, Gorn et al. 2002).

Signaling mechanisms

The instructive and permissive cues in the environment have to be read and interpreted correctly by the growth-cone, a specialized and highly motile structure at the tip of the growing axon. Each growth cone gets directed through binding the appropriate ligands determined through a specified set of receptors which regulates the cytoskeletal dynamics through secondary messengers (reviewed in (Tessier-Lavigne and Goodman 1996; Dickson 2002)).

Cytoskeleton

The cytoskeleton in the growth cone consists of microtubules and actin filaments and the reorganization and dynamics of these molecules causes the growth-cone to advance, retract, turn or branch (Dent and Gertler 2003). Stable bundles of microtubuli extend from the axon shaft and in the periphery of the growth-cone actin-filaments dominate. F-actin can either form the lamellipodium, a loose, interwoven network or it gets bundled into the filopodia which protrude from the growth-cone (Figure 4) (Lewis and Bridgmann 1992; Tanaka and Sabry 1995). Filopodia are essential for sensing guidance cues and steering the growth-cone since actin filaments play a central role in cell motility and are a direct target of guidance cues (Pollard and Borisy 2003). But also microtubules and their dynamics are important for growth-cone steering since they are linked to the actin filaments and some peripheral microtubules can even actively explore the periphery (Rodriguez, Schaefer et al. 2003). Molecules targeting the cytoskeleton are the downstream signal transduction units of receptors, activated by the different ligands (Tessier-Lavigne and Goodman 1996; Dickson 2002; Dent, Barnes et al. 2004) .

Secondary messengers

One important group of secondary messengers is the group of the Rho guanosine triphosphatases (GTPases) and their regulating proteins, the GTPase activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs). They have been identified in many organisms since they regulate cytoskeletal dynamics therefore influencing the advance or pruning of axons amongst many other tasks (Luo 2000; Hakeda-Suzuki, Ng et al. 2002; Kishore and Sundaram 2002; Ng, Nardine et al. 2002; Gitai, Yu et al. 2003). Growth-cone extension is caused by attractive cues through activation of Rac and Cdc42. These promote actin polymerization in lamellipodia and filopodia. Growth-cone retraction is caused through Rho activity which is induced by repulsive cues. They decrease actin-polymerization and cause growth-cone retraction through actin-myosin contraction (Guan and Rao 2003). Despite of these defined pathways, the turning response of the growth-cone depends critically on the levels of cyclic nucleotides which can convert the response from repulsion to attraction (Song, Ming et al. 1998; Nishiyama, Hoshino et al. 2003).

Rho-GTPases and their regulators signal downstream of the main classes of axon-guidance molecules such as the EphA receptors (Shaman, Lin et al. 2001), the Plexins (Tamagnone, Artigiani et al. 1999; Rohm, Rahim et al. 2000; Swiercz, Kuner et al. 2002;

Vikis, Li et al. 2002), Netrins (Round and Stein 2007) and the Robo receptors (Wong, Ren et al. 2001; Fan, Labrador et al. 2003; Hu, Li et al. 2005). In Robo receptor signalling especially the Vilse/crGAPs binding to the cc2-sequence (Lundstrom, Gallio et al. 2004; Hu, Li et al. 2005) and srGAPs (Slit-Robo GAPs) binding to the cc3-sequence (Wong, Ren et al. 2001) have been identified.

Other molecules influencing the cytoskeleton are the Ena/VASP proteins. They mediate filament elongation to produce longer and un-branched filaments with less protrusive force through antagonizing capping proteins (Bear, Loureiro et al. 2000). Slit mediated repulsion by the Robo receptors depends partially on Ena/VASP proteins since Ena binds to cc1 and cc2 (Bashaw, Kidd et al. 2000). And the repulsive Netrin-receptors - the UNC5 receptors - might signal through the Ena/VASP proteins (Colavita and Culotti 1998).

Ca^{2+}- signalling is another influence in the modulation of the cytoskeleton and its regulators. For example, it seems that the attractive response of Netrin through the DCC-family depends on Ca^{2+} influx through the plasma membrane and Ca^{2+}-release from the intracellular stores (Hong, Nishiyama et al. 2000).

Some signalling pathways can be modulated by phosphorylation. One possible outcome upon phosphorylation is retraction – e.g. for the Robo receptors. The tyrosine kinase Abelson (Abl) binds to the cc3-sequence of Robo and phosphorylates the cc1-, the cc2-sequence and another tyrosine residue in vitro (Bashaw, Kidd et al. 2000). Another example is Sema3A which causes growth-cone collapse. This may involve a LIM kinase which downregulates the cofilin-activity, therefore de-polymerizing actin filaments (Aizawa, Wakatsuki et al. 2001). ADF/cofilin (actin depolymerising factor) is an actin associated protein that enhances the dynamics of actin (Gehler, Shaw et al. 2004). However, phosphorylation can also cause attraction since Netrin signalling seems to involve complexes with tyrosine kinases like PKT-2, Src and Fyn (Round and Stein 2007).

However, the intracellular signalling machinery with its many members is an integration point. It combines the diverse signals which emanate from the set of receptors on the growth-cone onto the dynamics of the cytoskeleton.

The Drosophila embryonic CNS

The *Drosophila* embryonic ventral CNS consists of a chain of simple segmental ganglia. Each ganglion is composed of pairs of neurons which arise from the neuro-epithelium. The cell bodies lie ventral and send their neuronal processes into the neuropil where they form an orthogonal array with stereotypic projections. Two longitudinal tracts flank the midline connecting anterior and posterior. In each of the tracts about 150 longitudinal axons are organized in 15 to 20 distinct bundles. In each of the segments two commissures connect the longitudinal tracts, one anterior and one posterior commissure. About 80- 90% of the axons cross to the contralateral side (and never recross) whereas 10- 20% stay ipsilateral (Figure 5) (Jacobs and Goodman 1989; Jacobs and Goodman 1989; Broadie, Sink et al. 1993). The midline consists of six glial cells per segment (Jacobs and Goodman 1989) and it is analogous to the floorplate in the vertebrate spinal cord. It serves as a line of division for the bilateral symmetric CNS (Colamarino and Tessier-Lavigne 1995; Lapteva, Nieda et al. 2001).

The determination of the unique position of a neuron along the medio-lateral and dorso-ventral axes starts with axogenesis at stage 12 and persists until stage 17 (Thomas, Bastiani et al. 1984). All growing neurons have to do a series of decisions (Figure 6). The earliest decision is whether to leave the CNS or to grow towards the midline. At the midline, crossing and therefore contralateral neurons separate from non-crossing, ipsilateral neurons. Right after that neurons have to determine their lateral position and therefore distance to the midline. The next decision is whether to grow into an anterior or posterior direction and finally finding the correct target (Thomas, Bastiani et al. 1984).

Midline crossing

Midline crossing needs a well controlled balance between attraction and repulsion. Several well conserved receptor-ligand pairs have been identified to influence the decision of crossing. The ligands get expressed and secreted by the midline glial cells and they provide both, attraction and repulsion. This constant information gets interpreted by a well defined set of receptors on the growth-cones. Some of these receptors are constantly expressed and others are restricted in time and space.

Attraction of growth-cones across the midline works through the Fra receptor and its redundant ligands NetA and NetB (Harris, Sabatelli et al. 1996; Mitchell, Doyle et al. 1996; Brankatschk and Dickson 2006). Losing members of the attractive system – either

the receptor or both ligands - leads to a thinning or loss of some commissures (Harris, Sabatelli et al. 1996) because the now unbalanced system favours repulsion to the enfeebled attraction. However, this phenotype indicates that there might be another positive system, enabling neurons to cross the midline in the absence of the attraction through Netrins.

The repulsive system for midline crossing is composed of the ligand Slit and the Robo-family members which are expressed early: Robo and Robo2. The evidence for these conclusions comes from loss- and gain-of-function studies (Seeger, Tear et al. 1993; Kidd, Brose et al. 1998; Kidd, Bland et al. 1999; Rajagopalan, Nicolas et al. 2000[b;] Simpson, Kidd et al. 2000[b]).

In a *robo* mutant all axons cross and recross the midline abundantly, giving the molecule its name: Roundabout (Figure 7D) (Seeger, Tear et al. 1993; Kidd, Brose et al. 1998). A *robo2* mutant displays a more subtle phenotype with less mistakes in the crossing of axons (Figure 7C) (Rajagopalan, Nicolas et al. 2000[b;] Simpson, Kidd et al. 2000[b]). The single mutants of *robo* and *robo2* reveal the so called anti-linger functions of Robo and Robo2 where each receptor on its own can push axons out of the midline after entering it. The residual Robo in the robo2 mutant seems to do better than the residual Robo2 in the robo mutant since a robo mutant CNS is stronger condensed as a *robo2* mutant CNS (Figure 7B versus 7C). In a *robo robo2* double mutant (Figure 7E) all axons stay at the midline as they do in *slit* mutants (Rajagopalan, Nicolas et al. 2000[b;] Simpson, Kidd et al. 2000[b]). Robo3 seems to have only a function in lateral positioning since the *robo3* mutant reveals no midline crossing phenotypes (Figure 7B) also, it is expressed at later stages than Robo and Robo2 (Rajagopalan, Nicolas et al. 2000[b;] Simpson, Kidd et al. 2000[b]).

The switch for the decision about whether or not to cross is made by Commissureless (Comm) through post-transcriptional regulation of Robo (Figure 8) (Seeger, Tear et al. 1993; Tear, Harris et al. 1996; Keleman, Rajagopalan et al. 2002). Comm is a small, single-pass transmembrane molecule with no reported catalytic activity (Seeger, Tear et al. 1993; Tear, Harris et al. 1996). It is expressed in crossing, but not in ipsilateral neurons and this is opposite to the Robo expression: low in crossing and high in non-crossing neurons. This mutual exclusive expression pattern and genetic epistasis experiments suggested that Comm antagonizes Robo. Growth-cones with high Comm and low Robo have a reduced repulsion and therefore can cross the midline (Tear, Harris et al. 1996; Keleman, Rajagopalan et al. 2002; McGovern and Seeger 2003). Later studies showed how Comm exerts its function. Comm is localized at the Golgi and in late

endosomes (Keleman, Rajagopalan et al. 2002; Myat, Henry et al. 2002) recruiting Robo directly to endosomal degradation and thereby inhibiting Robo from reaching the cell surface to enact its repulsive activity (Keleman, Rajagopalan et al. 2002; Keleman, Ribeiro et al. 2005). Due to the absence of Robo and the attraction through Netrins, axons make it across the midline. After crossing Robo gets up-regulated and thus, axons are repelled from the midline. Ipsilateral neurons never express Comm and – expressing Robo from the outset – can not cross the midline at all. In *comm* mutants, all neurons act like ipsilateral neurons, express Robo from the onset, do not cross the midline and show the phenotype which gave Comm its name: commissureless (Seeger, Tear et al. 1993).

Comm appeared only recently in evolution and a vertebrate Comm does not exist. But instead Robo-3/Rig-1 seems to function as an anti-Robo1 (Sabatier, Plump et al. 2004). First, it is expressed – like Comm and Robo in flies - in the opposite manner than Robo-1 and Robo-2 with high levels of Robo3/Rig-1 during crossing and low levels afterwards. Second, Robo-3$^{-/-}$ mice display a commissureless phenotype being dependent on Robo-1/Slit signalling. Third, in explant assays, pre-crossing Robo3$^{-/-}$ axons get repelled by Slit since they can not down-regulate Robo-1, whereas wildtype pre-crossing axons are insensitive to Slit (Sabatier, Plump et al. 2004). Fourth, a mutation in the human *robo3*-gene is involved in a syndrome (horizontal gaze palsy with progressive scoliosis – HGPPS) where certain motor and sensory axons can not cross the midline (Jen, Chan et al. 2004).

Lateral positioning

After having decided whether or not to cross the midline, an axon has to determine its position lateral to the midline. Early studies on the developing CNS of grasshopper embryos revealed that pioneer neurons find their distinct pathways very precisely and reliably, leading to the "labelled pathway" hypothesis. This hypothesis proposes that neuronal receptors guide the growth-cones towards and along unique surface-molecules of lateral pathways (Raper, Bastiani et al. 1983; Raper, Bastiani et al. 1983; Raper, Bastiani et al. 1983; Bastiani, Pearson et al. 1984; Goodman, Bastiani et al. 1984; Raper, Bastiani et al. 1984). But it became apparent that the identified short-range molecules – amongst others FasciclinII (FasII) and Sema1a (Bastiani, Harrelson et al. 1987; Patel, Snow et al. 1987; Harrelson and Goodman 1988; Kolodkin, Matthes et al. 1992; Nose, Mahajan et al. 1992; Kolodkin, Matthes et al. 1993) do not suffice to establish the sophisticated arrangement of the longitudinal tracts (Lin, Fetter et al. 1994). Later, models combining the short-range guidance of the pathway-labels and long-range guidance

through the Robo/Slit system were proposed (Rajagopalan, Vivancos et al. 2000[a;] Simpson, Bland et al. 2000[a]) .

Evidence for the Robo/Slit system being involved in lateral positioning comes from loss- and gain-of- function analyses, mainly using the above mentioned pathway label FasII on late stages of insect embryos. FasII is a homophilic cell adhesion molecule which labels four major fascicles out of ~20 main longitudinal fascicles. Three of these FasII positive fascicles lie in one plane and are spaced conveniently throughout the neuropil and therefore they have been widely used to assess phenotypes for lateral positioning. According to their position they are named medial, intermediate and lateral fascicle reflecting their distance to the midline (Figure 5). These fascicles were mainly used to visualize the “Robo-Code”.

Stainings against the individual Robo receptors reveal that each of the Robo-family members is expressed in a specific region of the CNS, forming three differentially labelled zones (Rajagopalan, Vivancos et al. 2000[a;] Simpson, Bland et al. 2000[a]). Robo is expressed throughout the entire width of the neuropil; Robo3 is expressed in the lateral two thirds and Robo2 only in the lateral one third (Figure 9). Like this, a Robo-only zone forms next to the midline represented by the medial fascicle. Adjacent to it, a Robo-Robo3 zone arises, which contains the intermediate fascicle. The lateral fascicle runs in the most lateral zone, where all three Robo receptors are expressed (Figure 10) (Rajagopalan, Vivancos et al. 2000[a;] Simpson, Bland et al. 2000[a]). This differential expression pattern fits with the results of loss- and gain of function studies of the Robo receptors. Robo seems to have little if any role in lateral positioning. A FasII-staining and the counterstaining with HRP (to show all axons) of a *robo* mutant reveals an abundantly crossing medial fascicle but no phenotype in the intermediate or lateral fascicle (Figure 11B) when compared to wildtype (Figure 11A) (Kidd, Brose et al. 1998; Simpson, Bland et al. 2000[a]). Misexpressing Robo in single axon markers which naturally express only Robo and run in the medial fascicle - the Apterous- (Ap-) neurons - could not shift them further away from the midline (compare Figure 11E: wildtype and Figure 11F: Robo-misexpresion) (Rajagopalan, Vivancos et al. 2000[a;] Simpson, Bland et al. 2000[a]). Robo3 in contrast has a very prominent lateral positioning function. The mutant embryos for *robo3* show a complete shift of the intermediate fascicle into the medial one (Figure 11D). Conversely, misexpressing Robo3 in the Ap-neurons shifts these further away from the midline and therefore away from the medial Robo-only into the intermediate Robo-Robo3 zone (Figure 11G) (Rajagopalan, Vivancos et al. 2000[a;] Simpson, Bland et al. 2000[a]). However, the *robo2* mutant displays – like in midline crossing - a more subtle phenotype.

About 25% of the axons of the lateral fascicles shift into the intermediate one and thus closer to the midline, also stalling and thus stopping of axons can be observed (Figure 11C) (Rajagopalan, Vivancos et al. 2000[a]; Simpson, Bland et al. 2000[a]). Even more, misexpression studies of Robo2 lead to two different hypotheses about the mechanism of lateral positioning. One favours a quantitative mechanism wherein the combined levels of Robo2 and Robo3 shift axons away from the midline. The more of any of the two receptors is expressed on the growth-cone, the further away from the midline an axon is positioned (Rajagopalan, Vivancos et al. 2000[a]). The other hypothesis proposes a combinatorial model where special features of each of the two receptors define the position of the axon (Rajagopalan, Vivancos et al. 2000[a]; Simpson, Bland et al. 2000[a]; Rajagopalan, Nicolas et al. 2000[b]; Simpson, Kidd et al. 2000[b]). The two hypotheses are based on different interpretations of the misexpression results of Robo2 in the Ap-neurons. One study (Rajagopalan, Vivancos et al. 2000[a]) reports a shift from the medial into the intermediate fascicle after the additional expression of Robo2 in Ap-neurons (Figure 11G), similarly to adding Robo3 in these neurons (Figure 11H). Misexpressing one copy of both, Robo2 and Robo3 - at the same time - shifts axons into the lateral and outermost fascicle (Figure 11I) showing that the two receptors cooperate in lateral positioning. This interpretation indicates that the two receptors should be inter-changeable in their lateral positioning function and that the mere quantitative amount of Robo2 and/or Robo3 is sufficient to define lateral positions. In the other study (Simpson, Bland et al. 2000[a]) Robo3 and Robo2 shift the Ap-neurons from their wildtype medial position into different lateral zones. Robo3-overexpression shifts them into the intermediate (Figure 11K) and Robo2-overexpression into the lateral zone (Figure 11J) thereby establishing a hypothesis based on a combinatorial code with distinct functions encoded in the protein domains of each Robo receptor. Robo3 defines the intermediate and Robo2 protein the lateral fascicle. A commonly accepted but yet unproven hypothesis is, that Slit is the cue which instructs the neurons - expressing their “Robo-Code” - about their lateral positions since it is assumed that Slit forms a gradient emanating from its source of production, the midline glial cells (Kidd, Brose et al. 1998; Kidd, Bland et al. 1999; Rajagopalan, Vivancos et al. 2000[a]; Simpson, Bland et al. 2000[a]).

Vertebrate lateral positioning seems to follow the same lines as in flies, thus showing a differential expression of Robo-1 and Robo-2 and mutant phenotypes being specific to certain regions. In mice, axons project into the ventral and lateral funiculi sorting themselves into their specific lateral positions. As observed in flies, Robo-1 and Robo-2 are mainly expressed on postcrossing axons and establish three different zones: a Robo-1 positive, medial zone, a Robo-1 and Robo-2 double-positive, intermediate and a Robo-

2 positive, lateral zone. Accordingly, Robo-1$^{-/-}$ embryos show an axonal shift from the medial into the lateral zone and Robo2$^{-/-}$ embryos the vice versa shift. But it is clear, that these mechanisms are much more complicated in mammals than in flies, being revealed by the amplified number of genes (*slit-1, slit-2* and *slit-3*), their redundant functions (even a Slit-triple mutant does not show a complete commissureless phenotype) and the presence of other attractants and repellents (Zou, Stoeckli et al. 2000; Charron, Stein et al. 2003; Long, Sabatier et al. 2004).

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Figure Legends

Figure 1. Schematic view of the *Drosophila* CNS representing the choice of either the anterior or the posterior commissure.
Derailed (Drl) positive axons (dark orange) can not invade Wnt5 (magenta) positive areas of the posterior commissure and thus grow into the anterior commissure. Only Drl negative axons (blue) are able to invade these posterior Wnt5 positive regions; anterior (a) posterior (p) (Zou 2004).

Figure 2. Structure of Fra/Dcc and Netrin.
Frazzled (Fra)/ Deleted in colorectal cancer (Dcc): yellow; Netrins: green. Ig: Immunoglobulin-domain; FN: Fibronectin III-domain; LamNT: Laminin N-terminal-domain; EGF: Epidermal growth factor; CT: C-terminal-domain.

Figure 3. The *Drosophila* Robo receptor family and Slit.
Robo (blue), Robo2 (red), Robo3 (pink) and Slit (orange). Ig: Immunoglobulin-like domain; FN: Fibronectin III-domain; cc0- cc3: conserved consensus sequences; LRR: leucine rich domains; EGF: Epidermal growth factor like-domain; LamG: LaminiG-domain; CT: Cystein knot at C-terminus.

Figure 4. Schematic view of the growth cone (Dickson 2002).
Location of actin derivates (orange) and microtubuls (blue) in the tip of a growing axon.

Figure 5. Wildtype CNS of a S16 *Drosophila* embryo.
Double-immunostaining: A staining with anti-HRP-Cy5 (magenta) shows all axons and thus, reveals the anterior and the posterior commissure. A staining against FasII (1D4) (green) visualizes the three exemplary longitudinal tracts of the ventral nerve cord: the medial- (m), the intermediate- (i) and the lateral-(l) fascicle. Yellow lines indicate one segment.

Figure 6. Schematic view of the *Drosophila* CNS.
The yellow rectangles represent the midline glia cells. The different choices at the midline are visualized by a non-crossing ipsilateral motoneuron (red) which leaves the CNS to innervate e.g. muscles, a non-crossing ipsilateral interneuron (green) and a crossing commissural interneuron (blue).

Figure 7. HRP-staining of the CNS, depicting different mutant phenotypes in midline crossing.

HRP-staining (magenta) reveals all neurons at the neuropil. In Wildtype (A) and *robo3* mutants (B) all commissures form fully. The degree of condensation increases starting from *robo2* mutants (C) via *robo* mutants (D) to *robo robo2* double mutants (E) where all axons collapse onto the midline.

Figure 8. Midline crossing is controlled by Comm.

Ipsilateral neurons (upper neuron in A and B) do not express Comm from the onset. Therefore Robo- levels are high on the growth-cone and axons are repelled from the midline as it is the source of Slit. Contralateral neurons (lower neuron in A and B) express Comm only while crossing (A). At this time, Robo can not reach the cell-surface of the growth-cone and thus, allows axons to enter the midline. But after crossing (B), Comm gets down- and Robo up-regulated allowing Robo to push the neurons out of the midline after they entered it (Keleman, Rajagopalan et al. 2002).

Figure 9. Expression patterns of the Robo receptors in S16 embryos.

Comparison of immunostainings against the respective receptor (light green) with a staining against all axons using anti-HRP-Cy5 (magenta) reveals that Robo (A) is expressed on all neurons of the longitudinal tracts, Robo3 (B) is only on the axons of the lateral two-thirds and Robo2 (C) only expressed in the lateral one-third of the neuropil.

Figure 10. The Robo-Code model.

The three Robo receptors (Robo, Robo2 and Robo3) specify three distinct zones of expression and are arranged on a proposed but never proven Slit gradient. Next to the midline forms a Robo-only zone (m: medial zone), in the intermediate zone (i: intermediate zone) axons are sorted which express Robo and Robo3 and the very lateral position (l: lateral zone) is chosen from axons with all three Robo receptors (Rajagopalan, Vivancos et al. 2000[a]).

Figure 11. Comparison of *robo, robo2, robo3* mutant, and Robo-, Robo2- and Robo3- misexpression phenotypes.

A to D) Staining against all neurons of the neuropil with anti-HRP-Cy5 (magenta) and against an ipsilateral subset of motoneurons (antiFasII, green) representing the three Robo-Code zones: medial (innermost fascicle), intermediate and lateral (outermost fascicle). In wildtype (A) three distinct, and non crossing FasII fascicles are visible and the

neuropil forms its typical ladder shaped structure. In *robo* mutants (B) the innermost fascicle crosses the midline abundantly (arrowhead) and the neuropil appears condensed. In *robo2* mutants (C) FasII-positive fascicles cross the midline (arrowhead) and lateral positioning phenotypes, especially of the lateral fascicle appear (arrow). A *robo3* mutant (D) looses its intermediate fascicle through fusion into the medial fascicle (arrow) while the overall structure seems to be preserved. E to F) (from (Rajagopalan, Vivancos et al. 2000[a])) Counterstaining against HRP-Cy5 (green) shows all neurons and red visualizes either the UAS-τ-lacZ positive (E) or the UAS-HA-Robo receptor positive neurons: UAS-HA-Robo (F), UAS-HA-Robo2 (G), UAS-HA-Robo3 (H) and one copy of both at the same time: UAS-HA-Robo2 and UAS-HA-Robo3 (I). Overlap is shown in yellow. Misexpression of Robo in the Ap-neurons does not shift them further lateral from their medial position (arrow in F) but misexpression of Robo2 (arrow in G) and Robo3 (arrow in H) singularly shifts them into an intermediate position and misexpression of both, Robo2 and Robo3 at once shifts them into a lateral position arrow in I). J and K) (from (Simpson, Bland et al. 2000[a])) Misexpressing Ap-neurons are shown in yellow, UAS-HA-Robo2 overexpression (J) shifts axons into the lateral zone (arrow) and UAS-HA-Robo3 misexpression (K) shifts them into the intermedial zone (arrow), in K BP102 is visualized in red.

Figures

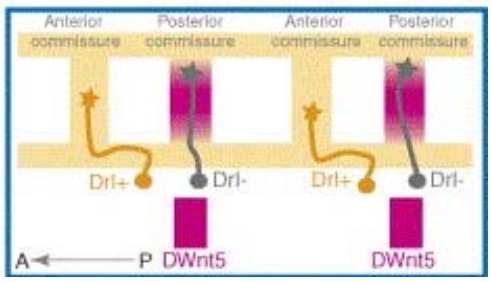
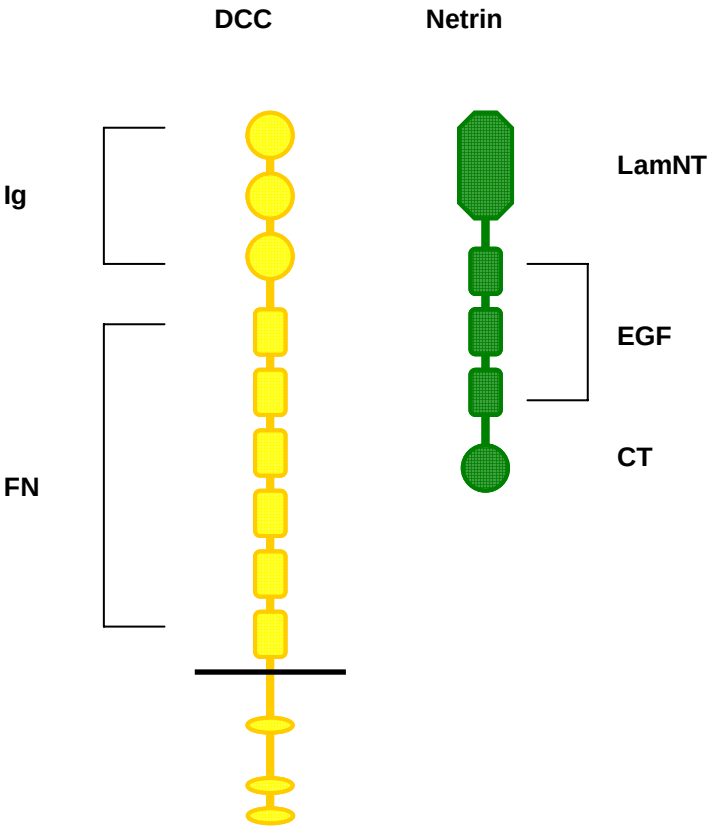


Figure 1



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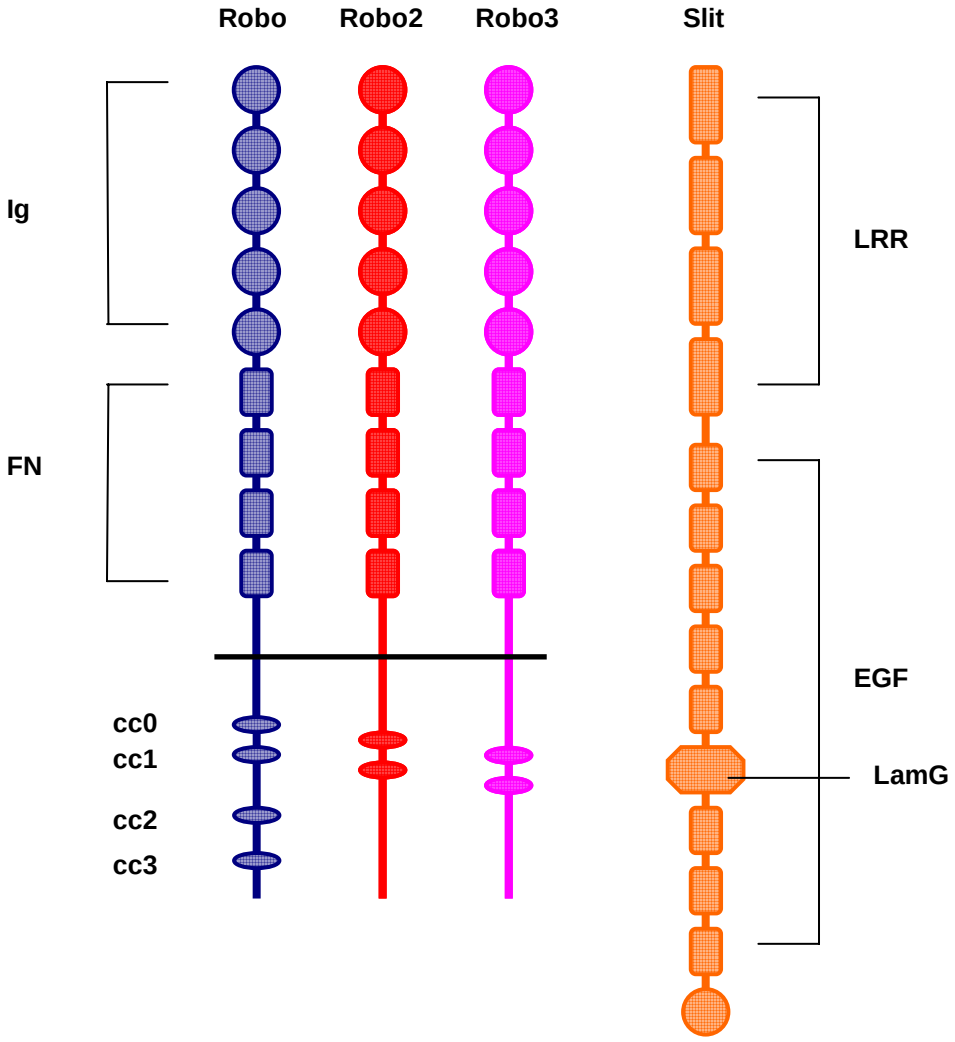

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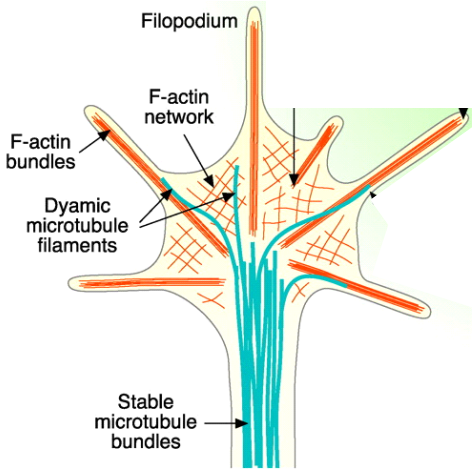
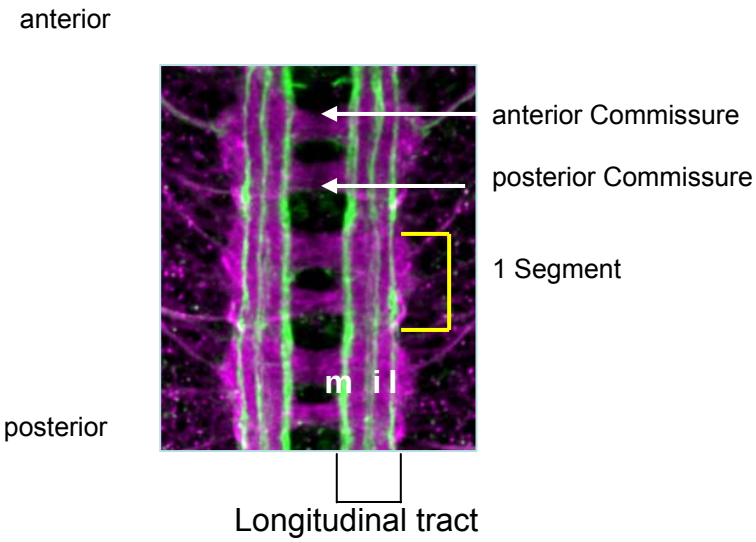


Figure 4



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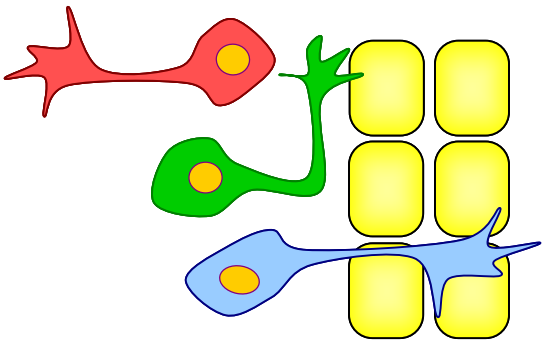
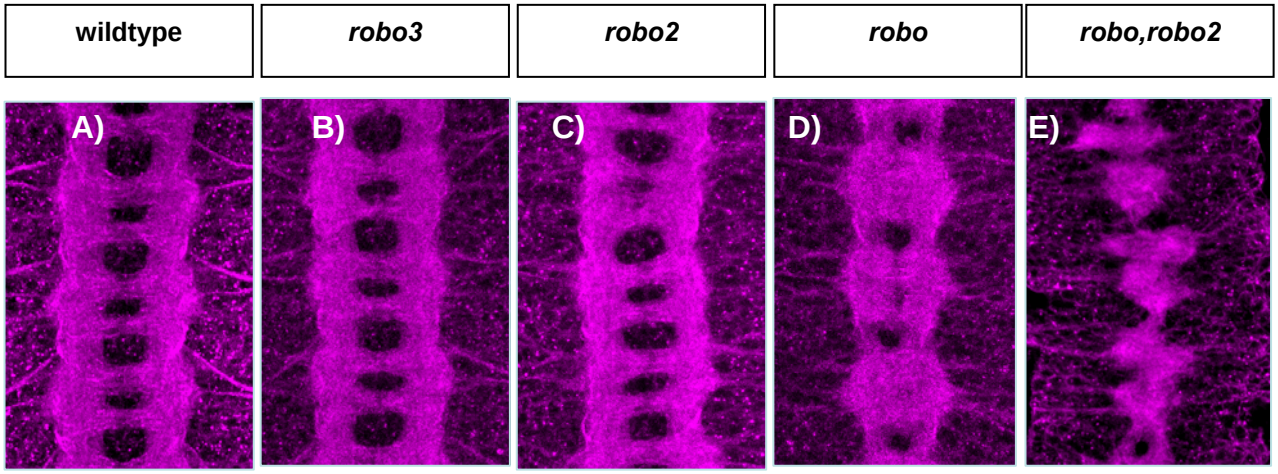


Figure 6



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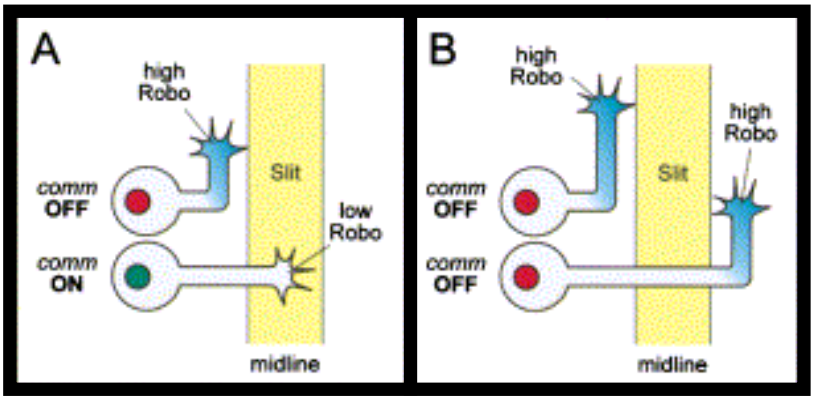

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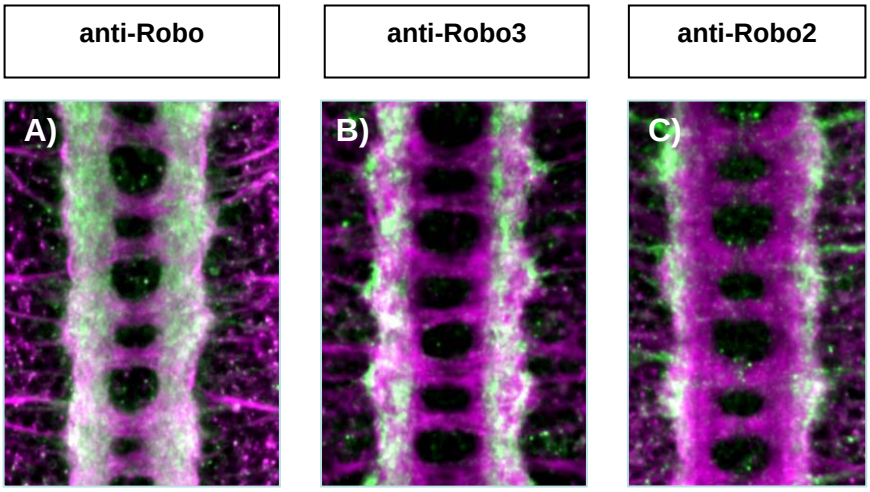

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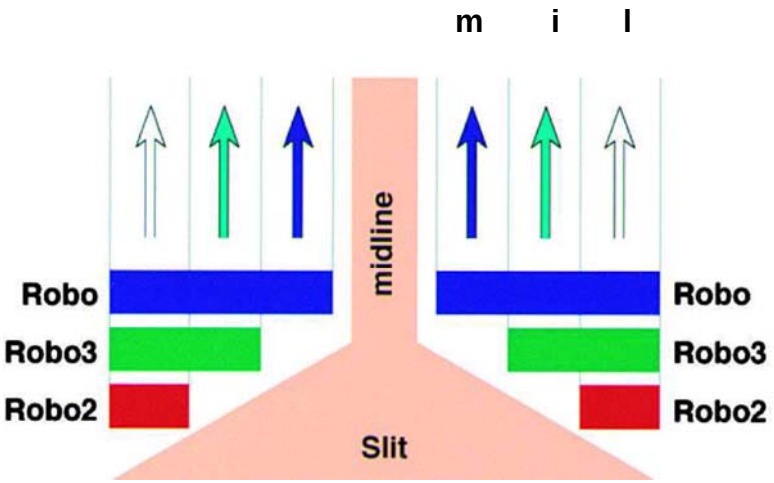
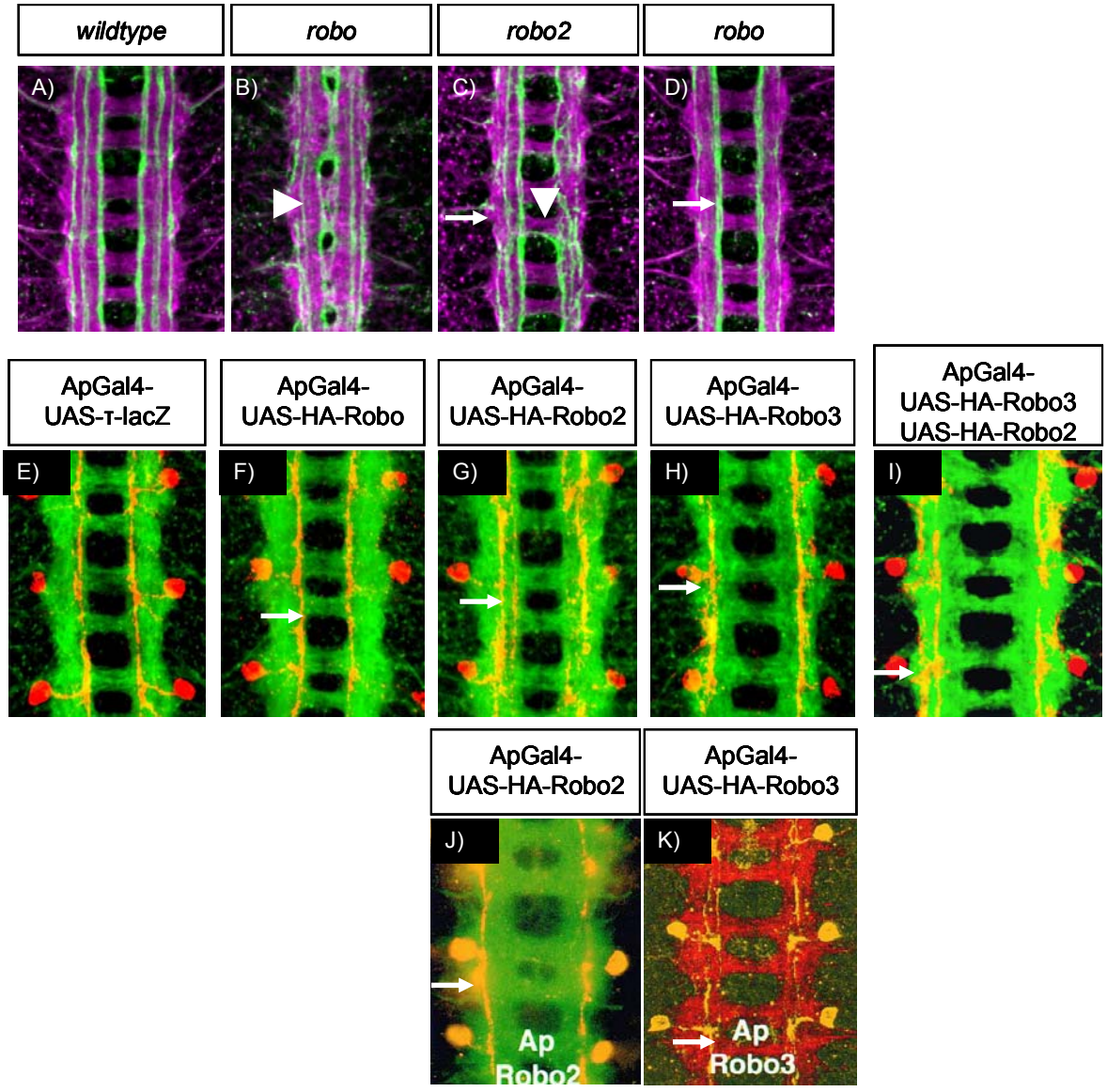


Figure 10


wildtype
robo
robo2
robo
A)
B)
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D)
ApGal4-
UAS-τ-lacZ
ApGal4-
UAS-HA-Robo
ApGal4-
UAS-HA-Robo2
ApGal4-
UAS-HA-Robo3
ApGal4-
UAS-HA-Robo3
UAS-HA-Robo2
E)
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Figure 11

Chapter 1 (Manuscript):

Functional specialization of Robo receptors in *Drosophila* axon guidance

Summary

The orthogonal array of axon pathways in the *Drosophila* CNS is constructed in part under the control of three Robo family axon guidance receptors, Robo1, Robo2 and Robo3. Each of these receptors is responsible for a distinct set of guidance decisions. To determine the molecular basis for these functional specializations, we used homologous recombination to create a series of 9 "*robo* swap" alleles: expressing each of the three Robo receptors from each of the three *robo* loci. We demonstrate that lateral positioning of the longitudinal axon pathways relies on differences in *robo* gene regulation, not distinct combinations of Robo proteins as previously thought. In contrast, specific features of the Robo1 and Robo2 proteins contribute to their distinct functions in commissure formation. These specializations allow Robo1 to prevent crossing, and Robo2 to promote crossing by antagonizing Robo1. These data demonstrate how diversification of expression and structure within a single family of guidance receptors can shape complex patterns of neuronal wiring.

Introduction

Complex neuronal wiring patterns emerge during development as individual axons respond differently to the same set of extracellular guidance cues. A single guidance cue can thus trigger a variety of growth cone responses – attracting some axons while repelling others, or guiding some axons at a distance but others only upon direct contact with the source (Tessier-Lavigne and Goodman 1996). How can a single guidance factor elicit such diverse responses? This question is usually answered by pointing out that most guidance molecules have multiple different receptors, that distinct receptors mediate distinct cellular responses, and that receptor expression is highly regulated at both the transcriptional and post-transcriptional levels (Yu and Bargmann 2001; Dickson 2002). However, this answer begs a second question: How do the different receptors acquire their different functions? What, in other words, is the molecular basis for the diverse receptor functions that in turn ensure that different growth cones respond differently to their common ligand? We explore this question here, focusing on the role of

the Roundabout (Robo) family receptors in patterning axonal projections in the nerve cord of the *Drosophila* embryo (Dickson and Gilestro 2006).

Most axonal growth cones in the *Drosophila* ventral nerve cord initially extend toward the midline. Some stop or turn longitudinally before they reach the midline, but most continue across to the contralateral side. Those axons that cross form the commissures that connect the two symmetric halves of the nervous system. Upon reaching the contralateral sides, many of these commissural axons then turn longitudinally, extending parallel to the midline but never recrossing it. Within the longitudinal pathways, axons are organized into a series of discrete fascicles, each located at a characteristic position lateral to the midline.

These various axonal trajectories are thought to be guided to a large extent by two factors produced by cells at the midline: Netrin (Harris, Sabatelli et al. 1996; Mitchell, Doyle et al. 1996) and Slit (Kidd, Bland et al. 1999). Netrin acts as a short-range attractant for commissural axons (Harris, Sabatelli et al. 1996; Mitchell, Doyle et al. 1996; Brankatschk and Dickson 2006), signalling through the DCC family receptor Frazzled (Fra) (Kolodziej, Timpe et al. 1996). Slit, in contrast, is thought to act primarily as a repellent for CNS axons (Kidd, Bland et al. 1999), signalling through various combinations of the three Robo family receptors: Robo, Robo2, and Robo3. Here, for clarity, we refer to Robo (the founding member of the family) as Robo1, using Robo as a generic name for the family.

The initial decision to cross or not to cross the midline is primarily controlled by Robo1 and its negative regulator Comm. Both ipsilateral and commissural axons express Robo1 (Kidd, Brose et al. 1998), but only commissural axons express Comm (Keleman, Rajagopalan et al. 2002). In these neurons, Comm appears to function as an endosomal sorting receptor that prevents most Robo1 from reaching the axonal growth cone, thereby rendering these axons insensitive to the Slit repellent (Keleman, Rajagopalan et al. 2002; Myat, Henry et al. 2002; Keleman, Ribeiro et al. 2005). Crossing itself requires in part Netrin signalling through Fra (Harris, Sabatelli et al. 1996; Kolodziej, Timpe et al. 1996; Mitchell, Doyle et al. 1996). However, many axons do still cross in both *Netrin* and *fra* mutant embryos (Harris, Sabatelli et al. 1996; Kolodziej, Timpe et al. 1996; Mitchell, Doyle et al. 1996), and commissural axons still orient their initial growth toward the midline in *Netrin* mutants (Brankatschk and Dickson 2006). For these reasons, it has long been assumed that some other factors act alongside Netrin and Fra to promote crossing. These factors have not yet been identified.

Although initially attracted to the midline, commissural axons do not stop once they reach it. Instead, they continue through to the contralateral side. However, in embryos lacking *slit* function (Kidd, Bland et al. 1999), or both *robo1* and *robo2 (Rajagopalan, Nicolas et al. 2000[b;] Simpson, Kidd et al. 2000[b])*, axons do remain at the midline. This suggests that axons are driven through the midline by Slit acting through the low levels of Robo1 and Robo2 that escape the sorting action of Comm. After crossing, it is evidently Robo1 that prevents recrossing of these commissural axons, just as it prevents crossing of longitudinal axons (Seeger, Tear et al. 1993; Kidd, Brose et al. 1998). Some aberrant midline crossing also occurs in both *robo2* and *robo3* mutants, but at a much lower frequency (Rajagopalan, Nicolas et al. 2000[b;] Simpson, Kidd et al. 2000[b]). Thus, Robo1 is primarily responsible for preventing inappropriate midline crossing, while Robo1 and Robo2 act redundantly to keep commissural axons from lingering at the midline.

Whether or not they initially cross the midline, many axons subsequently extend longitudinally alongside the midline. These longitudinal axons are sorted into three lateral zones, each defined by a specific combination of Robo receptors (Rajagopalan, Vivancos et al. 2000[a;] Simpson, Bland et al. 2000[a]). Axons in the medial zone express only Robo1, those in the intermediate zone express both Robo1 and Robo3, and those in the most lateral zone express all three Robos. Genetic loss- and gain-of-function studies have demonstrated that the Robo receptors are instructive in lateral positioning, and thus constitute a "Robo code" (Rajagopalan, Vivancos et al. 2000[a;] Simpson, Bland et al. 2000[a]). For example, upon loss of *robo3*, axons of the intermediate zone shift into the medial zone. Conversely, forced expression of Robo3 in specific medial zone neurons shifts their axons laterally into the intermediate zone. Similarly, loss of *robo2* shifts some lateral axons medially, and forced expression of Robo2 also shifts medial axons laterally. In contrast, Robo1 expression does not discriminate between longitudinal pathways, suggesting that it does not contribute to lateral pathway selection. Indeed, three discrete longitudinal zones still form in *robo1* mutant embryos, and forced expression of Robo1 also does not shift medial axons laterally. Thus, it is primarily Robo3 and Robo2 that function in lateral positioning. They may do so in response to a gradient of Slit activity spreading laterally from the midline, although a role for Slit in lateral positioning has not been directly demonstrated (Rajagopalan, Vivancos et al. 2000[a;] Simpson, Bland et al. 2000[a]).

In summary, then, Robo1, Robo2 and Robo3 each have distinct functions in axon guidance in the ventral nerve cord: Robo1 prevents inappropriate crossing, Robo1 and

Robo2 together prevent lingering at the midline, and Robo2 and Robo3 specify the lateral positions of longitudinal axons. What determines these distinct functions? In part, this can be explained by differences in their expression patterns. For example, Robo3 does not appear to be expressed early enough to influence the initial crossing decisions of pioneer commissural and longitudinal axons, and differences in *robo* expression clearly contribute to the "Robo code" for lateral pathway selection. But are there also critical biochemical differences between the three Robo proteins? Such differences have been explicitly invoked to explain lateral positioning:

> "Robo3 and Robo2 must differ from one another either in their ectodomains (and thus their abilities to read the Slit gradient), or in their cytoplasmic domains (and thus have different abilities to signal), or both"(Simpson, Bland et al. 2000[a]).

Whether such differences indeed exist between the Robos, and to what extent such differences contribute to their various guidance functions, has not been resolved.

We have used homologous recombination to construct a set of 9 "*robo* swap" alleles, expressing each of the three Robo receptors in each of the three distinct spatial and temporal patterns of the three *robo* genes. Surprisingly, and in contrast to previous models (Rajagopalan, Vivancos et al. 2000[a]; Simpson, Bland et al. 2000[a]), we find that lateral positioning does not rely on biochemical differences between the Robo receptors – neither in their ectodomain nor cytoplasmic domains. Expression differences alone can account for lateral pathway selection. In contrast, biochemical differences are critical in the midline crossing decisions. Additionally, our experiments reveal a unique biochemical role for Robo2 in promoting rather than preventing crossing, most likely by antagonizing the function of Robo1. Using these Robo swap alleles, we have thus been able to demonstrate how diverse guidance functions arise within a single family of guidance receptors due to differences in both receptor structure and expression.

Results

The *robo* Swaps

The three Robo receptors differ in both their expression patterns and structure (Kidd, Brose et al. 1998; Rajagopalan, Vivancos et al. 2000[a]; Simpson, Bland et al. 2000[a]; Rajagopalan, Nicolas et al. 2000[b]; Simpson, Kidd et al. 2000[b]). Robo1 and Robo2 are expressed in most and possibly all neurons as the initial axon pathways are pioneered

during embryonic stage 12 (Figure 1A, left). Robo3 is not expressed until stage 13, and remains limited to a subset of neurons. Robo1 expression persists throughout embryogenesis, but Robo2 is extinguished in many neurons as development proceeds. Thus, from stage 14 onwards, most neurons express one of three specific combinations of Robo receptors, according to which longitudinal axons are sorted into one of three lateral zones (Figure 1A, right).

All three Robo receptors are single-pass transmembrane proteins. Their ectodomains are similar, each comprising a series of 5 fibronectin type III domains and 3 immunoglobulin domains. All three Robos bind Slit (Brose, Bland et al. 1999; Kidd, Bland et al. 1999) with similar affinity (Howitt, Clout et al. 2004). The Robos are more divergent in their cytoplasmic domains. Robo1 contains the four short sequence motifs (CC0-CC3) that are also found in most other Robo proteins in other species, but Robo2 and Robo3 lack the CC2 and CC3 motifs. The primary goal of the present study was to determine which of these differences – expression or structure – account for the distinct functions of each of the three Robos in axon guidance.

Our general strategy was to create a set of 9 *robo* swap alleles, each driving the expression of one Robo receptor in the spatial and temporal pattern of another (or itself as a control). We modified each of the *robo* loci by gene targeting (Rong and Golic 2000), replacing the exons that encode the mature protein, as well the small intervening introns, with a single exon encoding a full length Robo protein (Figure 1B). The replacement exon also introduced 3 tandem HA epitope tags at the amino terminus, allowing us to use the same anti-HA antibody to assess the distribution of each Robo swap protein. We refer to a specific *robo* swap allele as *robo*X^{roboY}, where X indicates the targeted locus and Y indicates the substituted coding region. For example, *robo1*^{robo2} is the knock-in of the *robo2* coding region into the *robo1* locus.

We verified the molecular structure of each of these 9 swap alleles by genomic PCR and DNA sequencing, and by staining ventral nerve cords of these embryos with anti-HA to visualize the knock-in proteins. We confirmed that the expression pattern of each HA-Robo protein perfectly matched the pattern of the endogenous Robo protein it replaced, both in stage 13 (Figure S1) and stage 16 (Figures 1C and 1D) embryos.

A potential pitfall of our strategy is that the deletion of most introns and/or the inclusion of the epitope tags could disrupt the function of one or other *robo* gene. To test this, we used anti-FasII mAb 1D4 to examine axonal pathways in the ventral nerve cord of each of

the three "iso-*robo*" alleles (in which a given *robo* was replaced with itself). In wild-type stage 16 embryos, anti-FasII labels several longitudinal fascicles on each side of the midline, appearing in dorsal views as 3 discrete pathways, one in each “Robo zone”. Each of the *robo* null mutants has a characteristic phenotype with this marker (Figure 1E). In *robo1* mutants, the medial pathways from each side of the midline are generally fused into a single pathway that meanders back and forth across the midline (Kidd, Brose et al. 1998), Figure 1E and Table 1). In *robo2* mutants, axons of the medial pathways cross in ~25% of segments, while the lateral fascicle is disrupted in ~35% of hemisegments ((Rajagopalan, Vivancos et al. 2000[a;] Simpson, Bland et al. 2000[a;] Simpson, Kidd et al. 2000[b]); Figure 1E and Table 1)). In *robo3* mutants, the intermediate fascicle is shifted medially in every hemisegment, fusing with the medial fascicle ((Rajagopalan, Vivancos et al. 2000[a;] Simpson, Bland et al. 2000[a;] Simpson, Kidd et al. 2000[b]); Figure 1F and Table 1). These phenotypes were not observed in any of the 3 iso-*robo* swap alleles (Figure 1F and Table 1). We also note that, whereas *robo1* and *robo2* null mutants are homozygous lethal, all three iso-*robo* swap alleles are viable and fertile as homozygotes. We thus conclude that the set of modifications that are common to all *robo* swap alleles do not interfere with *robo* function.

Lateral Pathway Selection Relies on Differences in Robo Expression

Using the “hetero-*robo*” swaps, we first asked whether lateral pathway selection depends on biochemical differences between the Robo proteins, as generally assumed (Rajagopalan, Vivancos et al. 2000[a;] Simpson, Bland et al. 2000[a;] Simpson, Kidd et al. 2000[b]), or possibly on differences in their expression patterns. If biochemical differences are indeed critical, the longitudinal pathways should be highly disorganized in each of the hetero-*robo* swap alleles. If it is only differences in expression pattern that matter, the longitudinal pathways should be normal. We focused on the role of *robo3*, since *robo3* loss-of-function embryos have a striking and highly penetrant phenotype in which axons of the intermediate zone appear to shift medially (Rajagopalan, Vivancos et al. 2000[a;] Simpson, Bland et al. 2000[a;] Simpson, Kidd et al. 2000[b]). In particular, the intermediate FasII pathway merges with the medial pathway in every hemisegment (Figure 2, Table 1).

Much to our surprise, in both *robo3*^{robo1} and *robo3*^{robo2} homozygous embryos, all three FasII pathways formed normally (Figure 2), with no significant differences in FasII pathway formation between either of these *robo3* swap embryos and the wild-type or *robo3*^{robo3} controls (Table 1). This result was particularly unexpected, as previous gain-of-function experiments had suggested that Robo1 could not direct axons into more lateral

(Rajagopalan, Vivancos et al. 2000[a;] Simpson, Bland et al. 2000[a;] Simpson, Kidd et al. 2000[b]). Note also that in *robo3*^{robo1} embryos, the medial and intermediate zone axons express the same Robo code (Robo1 only), and similarly in *robo3*^{robo2} embryos, the intermediate and lateral axons express the same Robo code (Robo1 + Robo2). Yet in both cases, the three Robo zones formed normally. These data suggest that the "Robo code" is a code of gene expression; it does not rely on the distinct combinations of Robo receptors present in the growth cone, but on their additive expression patterns.

The role of Robo2 in the formation of the lateral axon pathways is less clear. This lateral FasII fascicle is only partly disrupted in *robo2* mutants ((Rajagopalan, Vivancos et al. 2000[a;] Simpson, Bland et al. 2000[a;] Simpson, Kidd et al. 2000[b]), and the interpretation of this phenotype is complicated by the additional roles of Robo2 in midline crossing (Rajagopalan, Nicolas et al. 2000[b;] Simpson, Kidd et al. 2000[b]); see also below). We found that the defects in the lateral FasII fascicles were partially rescued in *robo2*^{robo3} embryos, but not at all in *robo2*^{robo1} embryos (Figure S2 and Table 1). However, these two swap alleles also fail to rescue other aspects of the *robo2* phenotype, as discussed below, and so these data do not necessarily imply a unique and direct role for Robo2 in positioning the most lateral longitudinal axons.

Unique Features of Robo1 Protein Prevent Midline Crossing

Robo1 has a critical role in preventing longitudinal axons from crossing the midline (Seeger, Tear et al. 1993; Kidd, Brose et al. 1998; Rajagopalan, Nicolas et al. 2000[b]). To test whether Robo2 and Robo3 could functionally substitute for Robo1, we examined midline crossing phenotypes in *robo1*^{robo2} and *robo1*^{robo3} homozygous embryos. *A priori*, we could envision several possible outcomes for this experiment. If the Robo2 and Robo3 proteins expressed from the *robo1* locus cannot be adequately downregulated by Comm, then commissural axons should be unable to cross the midline. Such a commissureless phenotype is observed when any of the three Robos is expressed from a strong pan-neuronal promoter (Rajagopalan, Nicolas et al. 2000[b;] Simpson, Kidd et al. 2000[b]). If, however, Robo2 and Robo3 are sufficiently downregulated, then we would predict either a wild-type phenotype or *robo1*-like phenotype, depending on whether or not they can substitute for Robo1 in preventing longitudinal axons from crossing.

In both *robo1*^{robo2} and *robo1*^{robo3} homozygous stage 16 embryos stained with anti-FasII, we observed midline crossing errors that were qualitatively (Figure 3A) and quantitatively (Table 1) similar to those observed in *robo1* null mutants. We also examined stage 13

embryos in order to follow the projections of the ipsilateral pioneer neuron pCC while crossing. In *robo1*^{robo2} and *robo1*^{robo3} embryos, as in *robo1* null mutants, the pCC axons projected aberrantly across the midline (Figure 3A). The misrouting of pCC is particularly telling, as its growth cone expresses both Robo1 and Robo2, yet it requires only Robo1 for its ipsilateral projection since there is no phenotype in a robo2 mutant (Kidd, Brose et al. 1998; Rajagopalan, Nicolas et al. 2000[b]). Thus, regardless of whether it is expressed from its endogenous locus or the *robo1* locus, Robo2 cannot prevent pCC from crossing. We conclude that midline repulsion of longitudinal axons requires features of the Robo1 protein that are not shared with either Robo2 or Robo3. We note however that both *robo1*^{robo2} and *robo1*^{robo3} homozygotes are viable and fertile, whereas *robo1* null mutants are lethal. This confirms that these alleles do express functional Robo proteins that can substitute for at least some functions of Robo1.

Is Robo1's expression pattern also relevant to its specific function in regulating midline crossing? To test this, we asked whether Robo1 could prevent inappropriate crossing even if it were provided exclusively from either the *robo2* or *robo3* locus. Specifically, we examined *robo1 robo2*^{robo1} / *robo1 robo2*$^{+}$ and *robo1 robo3*^{robo1} / *robo1 robo3*$^{+}$ embryos, which lack endogenous *robo1* function but instead express Robo1 in the pattern of either Robo2 or Robo3. In neither case did we observe any rescue of the *robo1* phenotype (Figure 3B). For the *robo3* locus, this was expected, as there is little *robo3* expression at the stage in which axon pathways are pioneered (Rajagopalan, Vivancos et al. 2000[a]; Simpson, Kidd et al. 2000[b]). For the *robo2* locus it was however somewhat surprising, as *robo1* and *robo2* have similar expression patterns at early stages (Rajagopalan, Vivancos et al. 2000[a]; Simpson, Kidd et al. 2000[b]). Thus, the specific role of Robo1 in preventing midline crossing can be attributed to both its unique pattern of expression as well as unique features of the Robo1 protein.

Midline crossing errors also occur in both *robo2* and *robo3* mutant embryos, albeit at a much lower frequency ((Rajagopalan, Nicolas et al. 2000[b]; Simpson, Kidd et al. 2000[b]); Table 1). For example, in *robo2* null mutant embryos, we observed FasII-positive axons crossing the midline in 23.7% of segments. This phenotype was observed in only 2.0% of segments in the *robo2*^{robo1} swap and 14.8% in the *robo2*^{robo3} swap (Table 1). Similarly, crossing errors were observed with anti-FasII in 4.1% of segments in *robo3* null mutants, but only 0.6% and 0.5% in the *robo3*^{robo1} and *robo3*^{robo2} swaps (Table 1). Thus, in contrast to Robo1, neither the Robo2 nor Robo3 protein has unique features that are required to prevent longitudinal axons from crossing.

Robo1 and Robo2 Function Differently to Keep Axons out of the Midline

In *slit* mutants, all axons converge upon the midline (Kidd, Bland et al. 1999). This phenotype is not observed in either *robo1* or *robo2* single mutants, but does occur in the *robo1 robo2* double mutant ((Rajagopalan, Nicolas et al. 2000[b;] Simpson, Kidd et al. 2000[b]); Figure 4). Accordingly, it has been suggested that Robo1 and Robo2 have identical and overlapping functions in preventing commissural axons from lingering at the midline (Rajagopalan, Nicolas et al. 2000[b;] Simpson, Kidd et al. 2000[b]). If this model is correct, the two receptors should be fully interchangeable in this function. One might further anticipate that Robo3 too could substitute for either Robo1 or Robo2 in this role. We tested these predictions by crossing each of the respective swap alleles into the *robo1 robo2* double mutant background.

In both *robo1*^{robo2} *robo2* and *robo1*^{robo3} *robo2* homozygote embryos, we observed the same midline collapse phenotype seen in the double null mutant (Figure 4). Thus, Robo1 cannot in fact be replaced by either Robo2 or Robo3. In contrast, both the *robo1 robo2*^{robo1} and *robo1 robo2*^{robo3} mutants had a milder phenotype (Figure 4). Both were somewhat variable, but *robo1 robo2*^{robo1} generally resembled the *robo1* single mutant phenotype, with little or no midline stalling, whereas *robo1 robo2*^{robo3} was generally intermediate between *robo1* single mutants and *robo1 robo2* double mutants (Figure 4). Thus, Robo2 can be fully substituted by Robo1 and partly by Robo3. We conclude that Robo1 and Robo2 act differently in preventing lingering, just as they do in preventing crossing. In both roles, Robo1 relies on biochemical properties that it does not share with either Robo2 or Robo3. In contrast, Robo2 can mediate midline repulsion using features common to all three Robos.

A Positive Role for Robo2 in Midline Crossing

Our *robo* swap alleles provided an excellent opportunity to explore other possible functions of Robo receptors in midline axon guidance. In particular, we wondered whether one or more of the Robos might additionally act as a positive factor in midline crossing. Altough Netrins have a key role in promoting midline crossing, many commissures do still form in embryos that lack the two Netrin genes, *NetA* and *NetB*, implying the existence of an additional positive system contributes to commissure formation (Harris, Sabatelli et al. 1996; Mitchell, Doyle et al. 1996; Brankatschk and Dickson 2006). Thus, for more than a decade, it has been clear that some other system might involve one of the Robos, and

that the prominent role of Robo receptors in midline repulsion may have hitherto obscured any additional role in promoting midline crossing. To test this prediction, we examined the consequence of removing one or more Robo receptors in embryos devoid of Netrin function ((*NetAB*; (Brankatschk and Dickson 2006)). *A priori*, the loss of a receptor for a midline repellent should, if anything, increase the number of commissures in the *NetAB* background. Thus, if fewer commissures were observed, this would be a strong indicator of a positive role for the missing Robo in commissure formation.

The phenotypes we observed in both *NetAB robo1* and *NetAB robo3* embryos appeared to be additive combinations of the respective *NetAB* and *robo* mutants (Figure 5A and Table 2). In contrast, *NetAB robo2* embryos had a surprising phenotype that could not be predicted from either single mutant. Whereas commissures are only mild disrupted in *NetAB* mutants, and normal or even excessive crossing occurs in *robo2* single mutants, commissures were almost completely eliminated in the *NetAB robo2* embryos (Figure 5A and Table 2). Removing pairs of *robo* genes in the *NetAB* background did not reveal any further synergistic interactions, other than the expected midline collapse phenotype in *NetAB robo1 robo2* embryos (Figure 5A). Thus, Robo2, and Robo2 alone, has an additional positive role in commissure formation. This positive role is also independent of Fra, because *NetAB fra* resemble *NetAB* (and *fra)* and *fra robo2* resembles *NetAB robo2* (Figure S3 and Table 2). Thus, Netrin-Fra and Robo2 act independently, and the positive role of Robo2 can not be explained by cross-talk between Robo2 and Fra (as proposed for their vertebrate counterparts (Stein and Tessier-Lavigne 2001).

We further used our *robo2* swap alleles to assess whether this unique positive role of Robo2 relies on unique biochemical properties. We crossed both *robo2*^{robo1} and *robo2*^{robo3} into the *NetAB* background, and found that neither Robo1 nor Robo3 can substitute for Robo2 (Figure 5B and Table 2). For example, the anterior commissure appeared normal in just 1.6% of segments in *NetAB robo2* embryos, 7.0% of segments in *NetAB robo2*^{robo1} embryos, and 6.2% of segments in *NetAB robo2*^{robo3} embryos (Table 2). In contrast, in control *NetAB robo2*^{robo2} embryos, the anterior commissure formed normally in 82.5% of segments, similar to the 80.4% of segments with a normal anterior commissure in *NetAB* embryos (Table 2). Thus, just as unique features of Robo1 prevent midline crossing, unique features of Robo2 promote midline crossing.

Robo2 Promotes Crossing by Antagonizing Robo1

We can envision two models to account for the positive role of Robo2 in midline crossing. In one scenario (Figure 6A, model 1), Robo2 transduces an attractive signal that promotes crossing, possibly in response to its midline ligand Slit. Such a model has previously been proposed for Robo2 in the guidance of ganglionic tracheal branches (Englund, Steneberg et al. 2002). Alternatively (Figure 6A, model 2), Robo2 might promote crossing by antagonizing the repulsive function of Robo1, thus mediating an “anti-repulsion” rather than an "attraction" signal. Formally, this model is analogous to the role of Comm in *Drosophila* (Seeger, Tear et al. 1993), and of Robo3/Rig-1 in vertebrates (Sabatier, Plump et al. 2004).

Distinguishing between these two possibilities requires asking whether Robo2 can still exert its positive role in the absence of Robo1. If Robo2 is an attractive guidance receptor (model 1) then loss of Robo2 should reduce commissure formation even in a *robo1* mutant background. Conversely, if Robo2 antagonizes Robo1-mediated repulsion (model 2) loss of Robo2 should have no effect in the *robo1* mutant background. This latter argument is analogous to the reasoning that initially defined Comm as a negative regulator of Robo1 (Seeger, Tear et al. 1993), before either gene had been identified: because the *comm robo1* double mutant resembled *robo1*, it was (correctly) concluded that Comm promotes crossing by antagonizing a repulsive function of Robo1 (Seeger, Tear et al. 1993).

There are two complications to performing a similar genetic test for Robo2’s positive role in midline crossing. First, this positive role is only revealed in the absence of Netrins, and so the epistasis experiment must be performed in the *NetAB* background. Second, because *robo2* has both positive and negative roles in midline crossing, we need to use an allele of *robo2* that specifically eliminates Robo2’s positive contribution to commissure formation. If we use an allele that also disrupts Robo2’s repulsive function, then the double mutant combination with *robo1* will simply produce the well-documented (but in this context uninformative) *slit*-like phenotype. Fortunately, our *robo2*^{robo1} swap is just such an allele. As we have shown, Robo1 can fully substitute for the repulsive functions of Robo2 (Figure 4 and Table 1), but not at all for its positive function (Figure 5 and Table 2). Note also that the *robo2*^{robo1} allele also does not rescue the *robo1* mutant (Figure 3B and Table 1), and so still allows us to test the epistatic interaction with the *robo1* mutant. Thus, we can discriminate between the "anti-repulsion" and "attraction" models by

examining the epistatic interaction between *robo2*^{robo1} and *robo1* in the *NetAB* background.

We found that the *NetAB robo1 robo2*^{robo1} phenotype qualitatively and quantitatively resembles *NetAB robo1* (Figure 6B and Table 2), with robust commissure formation and even ectopic midline crossing. It did not resemble the commissureless phenotype of *NetAB robo2*^{robo1} mutants, nor an intermediate between the two. For example, a thick anterior commissure was present in 61.4% of segments in *NetAB robo1 robo2*^{robo1} embryos and 79.5% of segments in *NetAB robo1* embryos, but only 7.0% of segments in *NetAB robo2*^{robo1} embryos (Table 2). Moreover, in both *NetAB robo1 robo2*^{robo1} and *NetAB robo1*, but not *NetAB robo2*^{robo1}, these commissures contain FasII-positive axons (Figure 6B). Thus, specifically disrupting *robo2*'s positive role has a dramatic effect in the *NetAB* background, but little consequence in the *NetAB robo1* background. These data are more readily explained by a model in which Robo2 promotes midline crossing by antagonizing the repulsive function of Robo1 (Figure 6A, model 2).

Discussion

The *robo* Swaps: Dissecting the Distinct Functions of Each Robo

The midline guidance cue Slit is thought to act through each of three different Robo family receptors to help form the orthogonal axonal pathways of the *Drosophila* ventral nerve cord. Each of the three Robos has a distinct role in forming these projections (Figure 7A). Robo1 is primarily required to prevent longitudinal axons from crossing the midline (Seeger, Tear et al. 1993; Kidd, Brose et al. 1998), and commissural axons from lingering at the midline (Rajagopalan, Nicolas et al. 2000[b;] Simpson, Kidd et al. 2000[b]). Robo2 plays a minor role in preventing longitudinal axons from crossing, acts redundantly with Robo1 to keep commissural axons moving through the midline (Rajagopalan, Nicolas et al. 2000[b;] Simpson, Kidd et al. 2000[b]), and, as we have shown here, facilitates crossing of commissural axons. Finally, Robo3 may also help prevent some longitudinal axons from crossing, but its major function is to direct the formation of the intermediate longitudinal pathways (Rajagopalan, Vivancos et al. 2000[a;] Simpson, Bland et al. 2000[a]).

The goal of this study was to assess whether each of these functional specializations reflects biochemical differences in the Robo proteins themselves, or differences in *robo* gene regulation. To this end, we used gene targeting to replace the coding region of each

robo gene with that of each other *robo*, creating a series of nine *robo* swap alleles. An intriguing pattern of specialization emerges, as summarized in Figure 7A. First, for all of its functions, Robo1 relies on unique biochemical features; it cannot be replaced by either Robo2 or Robo3. In contrast, both Robo2 and Robo3 can generally be substituted with one or both of the other Robos, although not always equally well. The notable exception is Robo2's role in promoting commissure formation, which requires unique features of the Robo2 protein.

A Robo Expression Code for Lateral Pathway Selection

In the longitudinal pathways, axons are organized into discrete and stereotyped fascicles. In part, this requires selective fasciculation, mediated by contact-dependent attractive or repulsive surface proteins that "label" specific axon fascicles (Goodman and Bastiani 1984). This includes the FasII protein we have exploited here as a marker (Lin, Fetter et al. 1994). In addition to these pathway labels, the lateral pathways are also segregated into three broad zones according to the distinct combination of Robo receptors they express (Rajagopalan, Vivancos et al. 2000[a;] Simpson, Bland et al. 2000[a]). Loss- and gain-of-function genetic experiments have shown that these Robo proteins are instructive in lateral pathway selection, and hence define a "Robo code" (Rajagopalan, Vivancos et al. 2000[a;] Simpson, Bland et al. 2000[a]).

A popular model for lateral pathway selection posits that the three Robo proteins have distinct distinct signalling properties, and that they position axons on a lateral gradient of their common ligand Slit, emanating from the midline (Rajagopalan, Vivancos et al. 2000[a;] Simpson, Bland et al. 2000[a]). In this model, the Robo proteins are assumed to differ in either their affinity for Slit, the strength of their "repulsive output", or both. As a result of such differences, Robo2 would repel longitudinal axons more strongly than Robo3 and Robo3 in turn more strongly than Robo1, thereby directly these axons to progressively more lateral zones. An alternative possibility is that the Robo proteins might instead act a homophilic adhesion molecules (Hivert, Liu et al. 2002). In such a model, the Robo proteins might act in a manner similar to other pathway labels such as FasII, but operate over broader zones. Regardless of whether they invoke a role for Slit, homophilic adhesion, or some other unidentified ligand, all models presented to date have invoked critical structural differences in the Robo proteins. Due to these structural differences, the three Robo proteins are thought to form a combinatorial code for lateral pathway selection.

Our data strongly suggest that this can not be the case – that lateral positioning does not rely on structural differences between the three Robo proteins. In particular, we find that lateral pathway selection works surprisingly well even when Robo3 protein is replaced by either Robo1 or Robo2. Although we cannot exclude some minor disruption in specific pathways, the overall structure of the longitudinal pathways appears normal in these embryos. Notably, this includes the formation of the intermediate FasII pathway, which was diagnostic for Robo3's role in lateral positioning (Rajagopalan, Vivancos et al. 2000[a]; Simpson, Bland et al. 2000[a]). Thus, for lateral positioning, the only relevant differences between the three Robos are in their patterns of gene expression. The Robo code is not a protein code, but a gene expression code.

At first glance, this result is difficult to reconcile with the previously published gain-of-function experiments (Rajagopalan, Vivancos et al. 2000[a]; Simpson, Bland et al. 2000[a]). In these experiments, the various Robo proteins were expressed from GAL4/UAS transgenes in specific neurons (the Ap neurons). These Ap neurons normally express only Robo1 and hence project ipsilaterally in the medial zone. In both reports, expression of Robo3 shifted these axons into the intermediate zone, as expected, but expression of Robo1 did not. Why might Robo1 be able to replace the endogenous Robo3 in our swap experiments, but not the transgenic Robo3 in these gain-of-function studies? A trivial, but unsatisfying, explanation is that this was an artefact of the GAL4/UAS system. It is notoriously difficult to control for the varying expression levels from different transgene insertions, and expression levels rarely match endogenous levels. More interesting possibilities are that the discrepancy may reflect differences resulting from assaying the behaviour of neurons that normally express Robo3 versus those that don't, or a "community effect" that is observed upon manipulating an entire cohort of neurons but not just a single neuron. In this regard it is also important to note that the Ap axons are likely to be follower, not pioneer, axons for their specific pathway. Whatever the reason for this discrepancy, the substitution of the *robo1* coding region into the *robo3* locus is presumably the more physiologically relevant assay.

How might differences in *robo* gene expression explain lateral positioning? One possibility is that it is only the total Robo level that is important, with higher levels sending axons further laterally on the presumptive Slit gradient. This model fits with the results of "supershifting" experiments, in which additional copies of the Robo3 transgene displaced the Ap axons even further from the midline (Rajagopalan, Vivancos et al. 2000[a]; Simpson, Bland et al. 2000[a]). It is also supported by mathematical modelling of the Robo code (Goodhill 2003). This model still invokes a role for the Slit gradient, for which there is

admittedly no direct evidence. Alternatively, lateral pathway selection might rely on critical differences in the precise spatial and temporal pattern of expression, rather than differences in total Robo levels.

Robo1 and Robo2 Proteins are Specialized for their Distinct Roles in Midline Crossing

It has long been appreciated that Robo1 is the primary receptor through which Slit repels longitudinal axons to prevent them from crossing the midline (Seeger, Tear et al. 1993; Kidd, Brose et al. 1998). Midline crossing errors occur in every segment of *robo1* mutants (Seeger, Tear et al. 1993), but relatively rare in both *robo2* and *robo3* mutants (Rajagopalan, Nicolas et al. 2000[b;] Simpson, Kidd et al. 2000[b]). These minor contributions from *robo2* and *robo3* have always seemed more likely to reflect differences in gene expression, as both *robo2* and *robo3* are expressed in fewer neurons than *robo1*, particularly during the later embryonic stages (Rajagopalan, Nicolas et al. 2000[b;] Simpson, Kidd et al. 2000[b]). Indeed, our swap experiments have shown that neither the *robo2* nor the *robo3* expression pattern can support the function of Robo1. Nonetheless, the reciprocal set of swap experiments also clearly demonstrates that Robo2 and Robo3 proteins are also biochemically incapable of providing the same repulsive function as Robo1. This may be because they lack the CC2 and CC3 motifs found in Robo1, as well as most Robo proteins in other species. In particular, CC2 is required for Robo1 function in transgenic rescue assays and acts as a docking site for Enabled (Bashaw, Kidd et al. 2000), a negative regulator of cell motility (Bear, Loureiro et al. 2000).

Not only is Robo2 evidently less potent in midline repulsion, our data suggest that it even acts positively to promote midline crossing. We assume that Robo2 normally exerts this function autonomously in commissural neurons, acting in parallel to Netrin-Frazzled signalling to allow midline crossing. Genetically, Robo2 appears to facilitate crossing by antagonizing the repulsive function of Robo1. Formally, this is analogous to the role of Comm in *Drosophila* (Seeger, Tear et al. 1993) and Robo3/Rig-1 in mice (Sabatier, Plump et al. 2004). Comm antagonizes Robo1 by blocking its insertion in the growth cone membrane, and instead diverting it for lysosomal degradation (Keleman, Rajagopalan et al. 2002; Myat, Henry et al. 2002; Keleman, Ribeiro et al. 2005). This mechanism is unlikely to apply to Robo2, as neither loss nor gain of *robo2* function appears to alter Robo1 levels (S. Rajagopalan and B.J.D., unpublished data). Furthermore, in cell culture experiments analogous to those performed with Comm (Keleman, Rajagopalan et al. 2002; Myat, Henry et al. 2002; Keleman, Ribeiro et al. 2005), co-expression of Robo2

does not alter the membrane localization of Robo1 (S. Rajagopalan and B.J.D., unpublished data). Similar lines of evidence suggest that mouse Robo3 also does not use such a mechanism, although here too the precise mechanism is unknown (Sabatier, Plump et al. 2004).

We suspect that *Drosophila* Robo2 and mouse Robo3 may act in a similar way to inhibit the respective Robo1's. Although our genetic experiments cannot resolve the mechanism, they do limit the possibilities. In particular, we note that unique features of the Robo2 protein are required. This argues against a model in which Robo2 might act as a "sink" to titrate Slit or any other common binding partner away from Robo1. If this were the case, *Drosophila* Robo3 should be able to substitute for Robo2. A more plausible scenario might involve a direct interaction between Robo2 and Robo1, mediated by residues of Robo2 that are not conserved in the other Robos. Heteromeric Robo complexes have been detected in vitro (Simpson, Kidd et al. 2000[b;] Hivert, Liu et al. 2002), but whether they also form in vivo, and if so how this might affect Robo1 function, remain open questions.

We now know of three factors that promote midline crossing: Comm, Netrin-Frazzled, and Robo2. Of these, only Comm appears to be instructive. It is expressed in commissural but not ipsilateral neurons (Keleman, Rajagopalan et al. 2002), and is both necessary (Seeger, Tear et al. 1993) and sufficient (Bonkowsky, Yoshikawa et al. 1999) for crossing. In contrast, both Frazzled and Robo2 are expressed in both commissural and ipsilateral neurons, and are required but not sufficient for crossing (Kolodziej, Timpe et al. 1996; Rajagopalan, Nicolas et al. 2000[b;] Simpson, Kidd et al. 2000[b]). These two factors are therefore permissive rather than instructive. They are also partially redundant and independent, as crossing is severely disrupted only when both are eliminated. A conceptual model for midline crossing (Dickson and Gilestro 2006) proposes a bistable switch created by the mutual inhibition between high Robo1 levels and midline crossing (Figure 7B). In such a model, Netrin-Frazzled and Robo2 may act in different ways to ensure the appropriate balance between midline attraction and midline repulsion, bringing this feedback loop into the dynamic range at which Comm can operate (Figure 7B). In principle, any one of the three factors (Comm, Robo2, or Frazzled) could have taken on the instructive role. Comm has evidently done so in *Drosophila*. To the extent that a similar feedback loop operates in mice, the instructive role may have fallen in this species to the Robo2 analog, Robo3 (Sabatier, Plump et al. 2004).

Material and methods

Generation of *robo* Swap Alleles

Each of the 9 *robo* swap alleles was generated by ends-in homologous recombination (Rong and Golic 2000). Nine donor constructs were prepared in P-element vectors, each containing a single exon encoding the desired HA-tagged Robo protein in the correct reading frame for fusion with the signal sequence encoded in the first exon of each *robo* gene. This replacement exon was flanked on the 5' side by 7.5–7.7 kb of genomic DNA from the locus to be targeted, and 1.4–1.9kb on the 3' side. An I-SceI site was included roughly in the middle of the 5' homology region. Two I-CreI sites were inserted at the distal end of the 3' homology region, separated by a mini-*white* marker. The entire targeting cassette was flanked by FRT sites. These constructs were prepared using standard PCR-based cloning procedures, using genomic DNA from the w^{1118} strain and plasmids containing the *robo1*, *robo2*, or *robo3* cDNAs as templates. All coding regions and cloning junctions were confirmed by DNA sequencing. Transgene insertions on the X or 3rd chromosome were used for targeting, as all three *robo* genes are located on the 2nd chromosome.

The targeting fragment was then liberated and linearized in the female germline using FLP and I-SceI, respectively, and progeny were screened for movement of the mini-*white* marker to the 2nd chromosome, as well as its resistance to *ey*FLP (indicating that it is no longer flanked by FRT sites, as in the donor; (Newsome, Asling et al. 2000). The successful generation of 1–6 homologous recombinants per allele was initially confirmed using a set of specific PCRs to detect the insertion of the replacement *robo* sequence and the disruption of the endogenous locus. The initial recombinants contained a duplication at the intended locus, which was subsequently resolved by using I-CreI to induce a double-stranded break and selecting in the progeny for the loss of the intervening $white^{+}$ marker. These recombinants were then screened by PCR to identify those that retained the replacement allele and had lost the endogenous allele, prior to the more extensive histological characterisation as described in the Results.

Immunohistochemistry

Immunofluorescence stainings of staged and fixed embryos were performed as described (Patel 1994). Robo (mouse), Robo2 (rabbit) and Robo3 (mouse) antisera were used at a

dilution of 1:1000, 1:200 and 1:500 respectively (Rajagopalan, Vivancos et al. 2000[a]). Other primary antibodies used were anti-FasII mAb 1D4 (1:1000, (Vactor, Sink et al. 1993), anti-HA mAb 3F10 (1:750, Roche Diagnostics), anti-HA mAb 16B12 (1:1000, BAbCO, Berkeley Antibody Company), anti-ß-galactosidase (1:1000, Promega) and Cy5-conjugated sheep anti-HRP (1:500, Jackson Immunoresearch). Secondary antibodies used were anti-mouse Alexa Fluor-488, anti-rat and anti-rabbit Alexa Fluor-568-conjugated (1: 1000, Molecular Probes). Homozygous embryos were identified by selecting against anti-ß-galactosidase staining indicating the presence of CyO, P[*wg-lacZ*] the balancer chromosome. Selected embryos were dissected, mounted in Vectashield mounting medium (Vector Labs) and images were acquired with a Zeiss LSM 510 confocal microscope.

Quantification of Midline Crossing and Lateral Positioning defects

For the quantification of defects in the commissural or longitudinal axon pathways, stage 16 or 17 embryos were stained with mAb BP102 or anti-FasII, respectively, using the Vectastain Elite ABC kit. Anti-ß-galactosidase was included to allow identification of the mutant embryos, which were dissected and mounted in 70% glycerol. Phenotypes were scored blind to the genotype using Nomarski optics on a Zeiss Axioplan 2 microscope with a 100 X objective.

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Figure Legends

Table 1. Midline Crossing and Longitudinal Pathway Errors in *robo* Swap Mutants.
Stage 16-17 embryos stained with anti-FasII were scored for the presence of FasII-positive axons extending across or along the midline, and for breaks in the intermediate and lateral FasII fascicle, often due to fusion with the more medial fascicle. Indicated are the genotype, viability, number of hemisegments scored, percentage of segments with FasII positive axons at the midline and percentage of mistakes in lateral positioning each for the medial (m), the intermediate (i) and the lateral (l) fascicle.

Table 2. Commissure Formation in *NetAB, fra* and *robo* Swap Mutants.
Stage 16-17 embryos with BP102 were scored for defects in the anterior and posterior commissures. Data in italics indicates a *robo1* mutant phenotype, for which "normal" also includes thicker commissures. The *robo1*^{1}*, robo1*^{8}*, robo2*^{4} *and robo2*^{8} alleles are null, and *robo3*^{1} is a strong hypomorph.

Figure 1. The *robo* Swaps
(A) Schematic of Robo expression patterns in representative neurons during the initial (stage 12, left) and later (stage 14-15, right) stages of axon pathfinding, adapted from (Simpson, Bland et al. 2000[a]) and (Rajagopalan, Vivancos et al. 2000[a]). M, I, and L indicate the medial, intermediate, and lateral zones, respectively, as defined by the combinatorial expression of the three Robos.
(B) Strategy used to construct the three *robo1* swap alleles. The *robo2* and *robo3* swaps were generated in an analogous fashion by targeting the respective genomic loci. SP indicates the exon encoding the signal peptide, which in all cases derives from the targeted locus.
(C) Nerve cords of wild-type stage 16 embryos stained with anti-Robo1, anti-Robo2, or anti-Robo3 (green). As in all similar images in this paper, embryos were counterstained with anti-HRP to visualize the neuropil (magenta), and confocal sections through the neuropilar region of three abdominal segments are shown with anterior up. Each Robo protein is largely excluded from commissures (arrows), but enriched across the entire longitudinal tract (Robo1) or a specific region of the longitudinal tract (Robo2 and Robo3; arrowheads) as indicated in (A).
(D) Stage 16 embryos of the indicated iso-*robo* swap allele stained with anti-HA to visualize the knock-in Robo protein (green). In each case, the substituted protein is expressed in a manner that matches that of the endogenous protein (C).

(E and F) Stage 16 embryos homozygous for the indicated *robo* mutation (E) or iso-*robo* swap (F), stained with anti-FasII (green). In the iso-*robo* swaps (F), as in wild-type embryos, FasII labels three longitudinal pathways, one in each "Robo zone" (A). In contrast, each *robo* mutant displays a characteristic phenotype: repeated crossing of the medial FasII pathway in the *robo1* mutant (left, arrows), occasional crossing errors (middle, arrow) and breaks in the lateral fascicle (middle, arrowhead) in the *robo2* mutant, and rare crossing (not shown) and a highly penetrant medial shift of the intermediate FasII fascicle (right, arrowheads) in the *robo3* mutant. These phenotypes were only rarely observed, if at all, in the iso-*robo* swaps (F, for quantification, see Table 1).

Figure 2. The Intermediate FasII Pathway is Normal in all three *robo3* Swap Mutants.

Stage 16 embryos of the indicated genotype, stained with anti-FasII (green). Note that the intermediate FasII-fascicle is shifted medially in the *robo3* mutant, but positioned normally in all three *robo3* swap alleles (arrowheads; for quantification, see Table 1). Schematics below each image indicate the Robo proteins expressed from each *robo* locus, according to the color scheme of Figure 1A.

Figure 3. Robo1 Function Requires Unique Structural Features and Expression.

Stage 16 (A and B) or stage 13 (A) embryos of the indicated genotypes, stained with anti-FasII (green). In stage 16 embryos, ectopic crossing of medial FasII-positive axons is observed in *robo1* mutants and all swap alleles with the exception of the *robo1*^{robo1} control (for quantification of genotypes in A, see Table 1). In stage 13 embryos, the pCC axons (arrowheads) project across the midline in *robo1* and each of the swaps alleles, except the *robo1*^{robo1} control.

Figure 4. Robo2, but not Robo1, is Replaceable as an "Anti-linger" Receptor.

Stage 16 embryos of the indicated genotypes, stained with anti-FasII (green). *robo1*^{robo2} *robo2*, *robo1*^{robo3} *robo2*, and, to a lesser extent, *robo1 robo2*^{robo3} all have midline collapse phenotypes almost identical to that observed in *robo1 robo2* mutants. Both *robo1 robo2*^{robo1} and *robo1 robo2*^{robo2} more closely resemble *robo1* mutants (Figure 1E). The nerve cord of the *robo1*^{robo1} *robo2* mutant appears slightly more disorganized than that of the *robo2* single mutant, suggesting that the *robo1*^{robo1} allele may be a very weak hypomorph (note also the normal appearance and full viability of *robo1*^{robo1} single mutants; Figures 1F and 3A and Table 1).

Figure 5. A Unique Role for Robo2 in Promoting Midline Crossing.

Stage 16 embryos of the indicated genotypes, stained with anti-FasII (green). Note the marked reduction in commissures in all combinations of *NetAB* together with a *robo2* allele (A), including the hetero-*robo2* swaps (B). By comparison, many commissures are still formed in the *NetAB* mutant, either alone or in combination with *robo1* or *robo3* (A), or with the *robo2*^{robo2} control allele (B). For quantification of phenotypes, see Table 2.

Figure 6. Robo2 Promotes Crossing by Antagonizing Robo1.

(A) Models for the function of Robo2 in promoting midline crossing, either by mediating midline attraction (model 1) or antagonizing midline repulsion mediated by Robo1 (model 2). Note that Robo2 also mediates midline repulsion in response to Slit (not shown), but this of little consequence in the initial crossing decision.

(B) Stage 16 embryos of the indicated genotypes, stained with anti-FasII (green). The *NetAB robo1 robo2*^{robo1} mutant resembles the *NetAB robo1* mutant, not *NetAB robo2*^{robo1}, with many axons crossing the midline. This includes FasII-positive axons that extend along or across the midline. For quantification, see Table 2.

Figure 7. Summary and Model for Commissure Formation

(A) Summary of data from the *robo* swap alleles. For each of the four functions, and each of the three *robo* genes, the table shows whether the Robo protein sequence is irreplaceable ("Unique"), or which other Robos can substitute if expressed at the same locus ("1", "2", and "3"), as well as how well they can substitute ("=" indicates full or almost full function, ">" indicates reduced function). A dash indicates that the *robo* gene is not required at all.

(B) Model for commissure formation, adapted and extended from (Dickson and Gilestro 2006). High Robo1 levels prevent crossing, and crossing leads to downregulation of Robo1 levels, thereby creating a bistable switch through mutual inhibition. This switch ensures a clear "cross / don't cross" decision for each axon. Comm, Robo2, and Frazzled each favor the crossing decision by modulating the feedback loop at different points, with Robo2 probably acting in a manner very similar to mammalian Robo3.

Figure S1. Expression of Iso-*robo* Alleles in Stage 13 Embryos

(A) Wild-type stage 13 embryos stained with anti-Robo1, anti-Robo2, or anti-Robo3 (green). Robo1 and Robo2 are similarly localized on longitudinal but not commissural pioneer axons at this stage; Robo3 is barely detectable.

(B) Stage 13 embryos of the indicated iso-*robo* swap allele stained with anti-HA to visualize the knock-in Robo protein (green). As in stage 16 embryos (Figure 1D), the

expression of the substituted protein also matches that of the endogenous protein at stage 13 (A).

Figure S2. Midline Crossing and Lateral Positioning in *robo2* Swap Alleles.
Stage 16 embryos of the indicated genotype, stained with anti-FasII (green). In *robo2* mutants, FasII-positive axons cross the midline in ~24% of segments (arrow), and the lateral FasII fascicle is disrupted in ~30% of hemisegments (arrowhead). The *robo2*^{robo1} swap rescues midline crossing defects but not the lateral fascicle defects, whereas *robo2*^{robo3} partially rescues both phenotypes. For quantification, see Table 1.

Figure S3. Robo2 and Frazzled Act Independently.
Stage 16 embryos of the indicated genotypes, stained with anti-FasII (green). For quantification of phenotypes, see Table 2.

Tables

Genotype	Hemi-segment	FasII positive axons at midline	Mistake of FasII fascicles (%)					
			m+i	i+l	m+i+l	Break l	Break i	Break m
wild type	610	0	0	0	0	0	0	0
robo1^{GA285}/ *robo1*^{Z3127}	288	100.0	0	0.7	0	0.7	0	0
robo1^{robo1}	272	0	0	5.9	0	0	0	0
robo1^{robo2}	330	97.6	0.9	3	0	1.2	0.3	0
robo1^{robo3}	264	99.2	0.8	2.6	0	1.1	0	0
robo2^{4}	186	23.7	10.2	29.9	0	10	1.1	2
robo2^{robo1}	300	2	0.7	33	0	4.3	0	0
robo2^{robo2}	308	0	0	1.0	0	0	0	0
robo2^{robo3}	284	14.8	2.2	13.7	0	6.7	0.4	0
robo3^{1}	340	4.1	100.0	0	0	0.9	0	0
robo3^{robo1}	266	0.6	0.4	3.9	0	0	0	0
robo3^{robo2}	384	0.5	0	1.0	0	0	0	0
robo3^{robo3}	304	0	0	1.7	0.3	0.7	0.3	0

Table 1

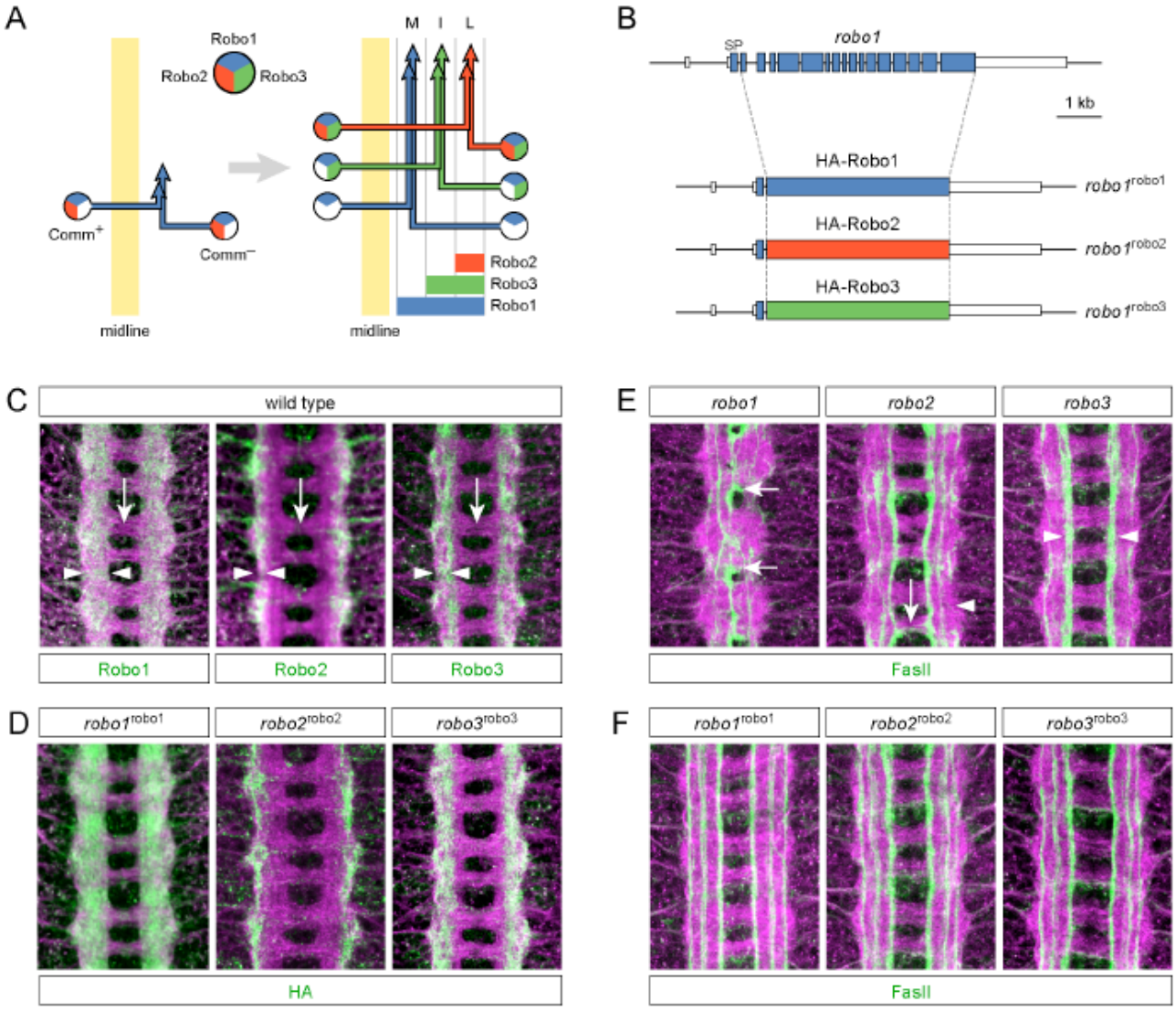
Genotype	Hemi-	Anterior Commissure			Posterior Commissure		
	segment	normal	thin	absent	normal	thin	absent
wild type	437	100	0	0	100	0	0
NetAB$^{\Delta}$	276	80.4	13.8	5.8	55.8	26.8	17.4
NetAB$^{\Delta}$*;robo1*^{GA957}*/*	154	79.5*	12.8	7.7	53.9*	14.5	31.6
NetAB$^{\Delta}$; *robo2*^{4}	247	1.6	27.6	70.7	0	12.9	87.1
NetAB$^{\Delta}$; *robo3*^{1}		88.0	6.5	5.4	60.9	23.9	15.2
NetAB$^{\Delta}$; *robo2*^{8}, *robo3*^{1}	150	4	34.7	61.3	6.7	9.3	84
fra^{23H9}*/fra*^{GA957}	222	93.7	5.4	0.9	80.2	13.5	6.3
fra^{23H9}*/fra*^{GA957}*;robo2*^{4}*/ robo2*^{8}	208	12.5	61.5	26.0	5.8	35.6	58.7
NetAB$^{\Delta}$; *robo2*^{robo1}	286	7.0	39.2	53.8	3.5	14	82.5
NetAB$^{\Delta}$; *robo2*^{robo2}	287	82.5	14	3.5	59.7	17.4	22.9
NetAB$^{\Delta}$; *robo2*^{robo3}	278	6.2	67.7	26.2	6.9	23.9	69.2
NetAB$^{\Delta}$*;robo1*^{GA285}, *robo2*^{robo1}	287	61,4*	22.1	16.4	25.9*	25.2	49

* *robo1*-like phenotype

** *slit*-like phenotype

Table 2

Figures



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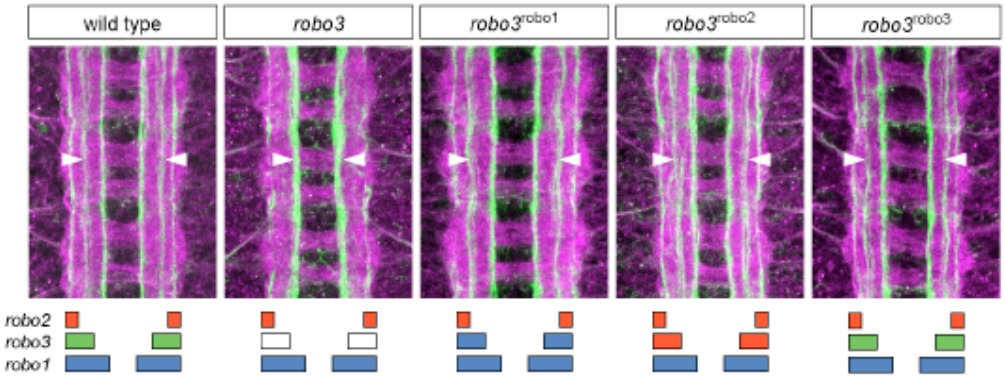


Figure 2

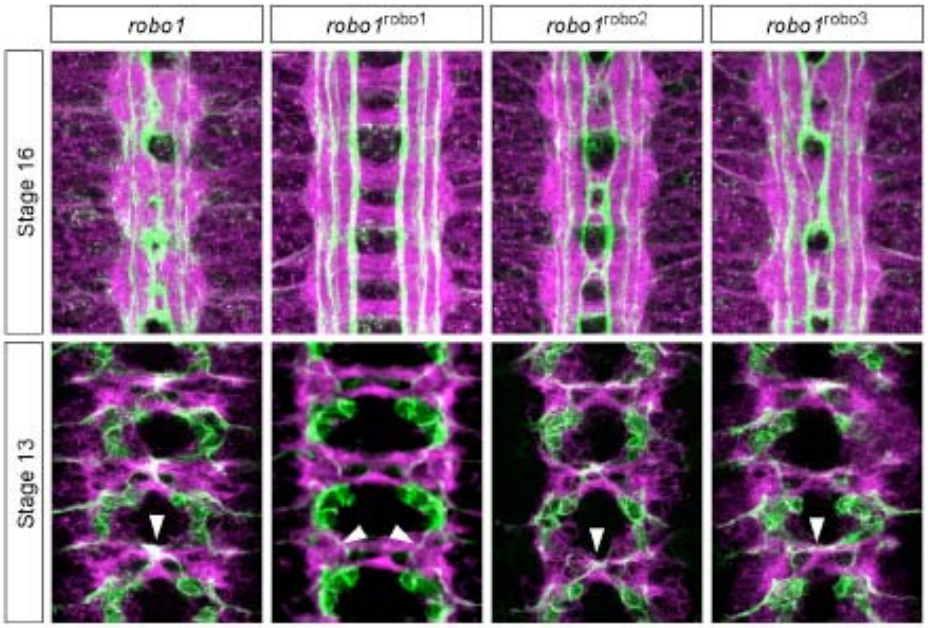


Figure 3

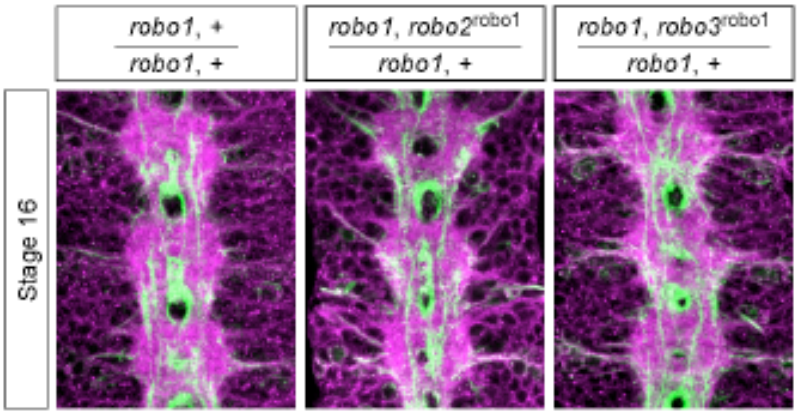
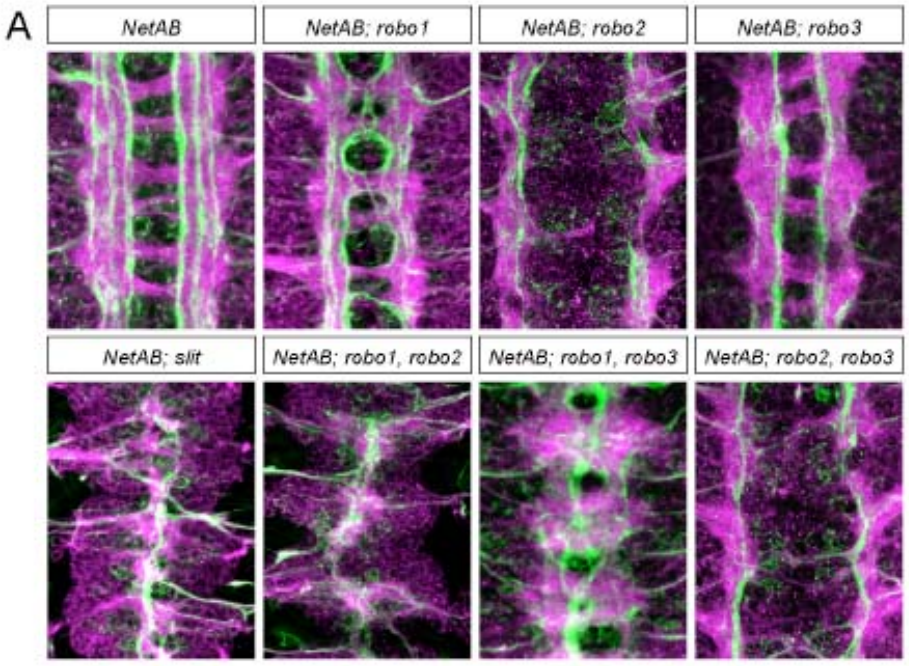


Figure 4



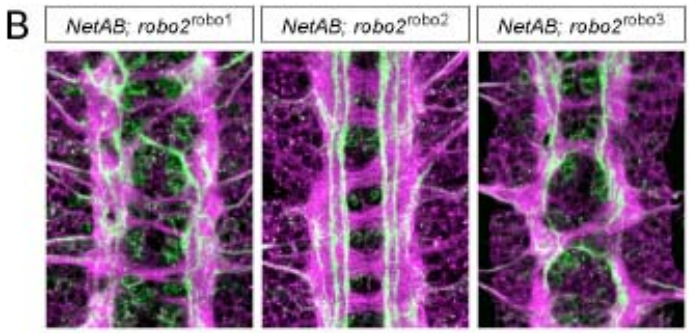


Figure 5

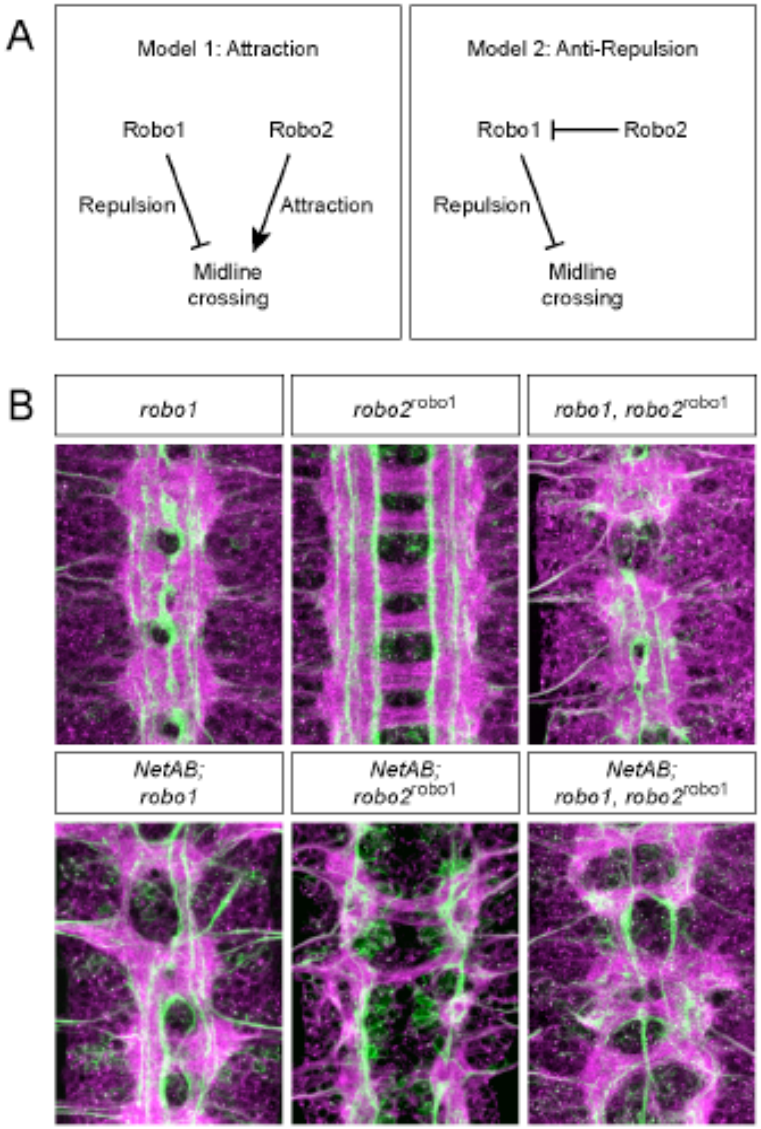

A
Model 1: Attraction
Robo1
Robo2
Repulsion
Attraction
Midline crossing
Model 2: Anti-Repulsion
Robo1
Robo2
Repulsion
Midline crossing
B
robo1
robo2robo1
robo1, robo2robo1
NetAB; robo1
NetAB; robo2robo1
NetAB; robo1, robo2robo1

Figure 6

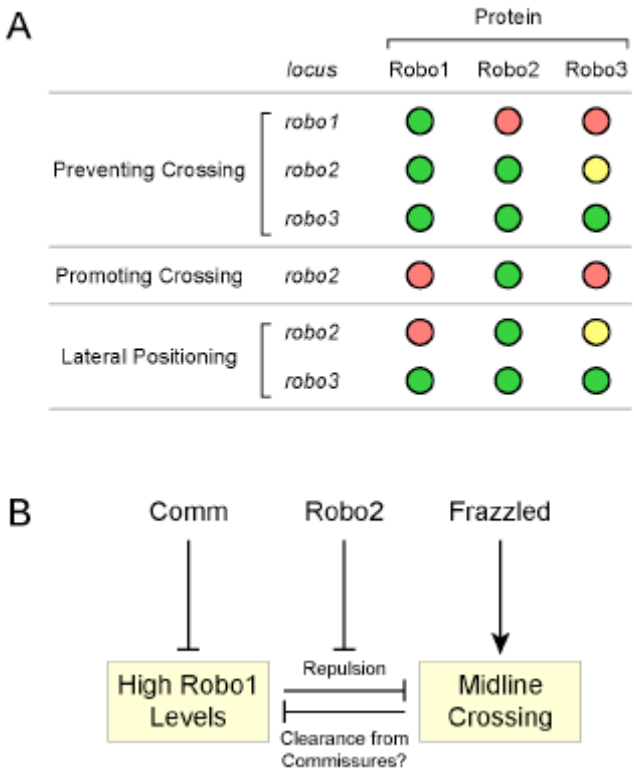

A
Protein
locus
Robo1
Robo2
Robo3
Preventing Crossing
robo1
robo2
robo3
Promoting Crossing
robo2
Lateral Positioning
robo2
robo3
B
Comm
Robo2
Frazzled
High Robo1 Levels
Repulsion
Clearance from Commissures?
Midline Crossing

Figure 7

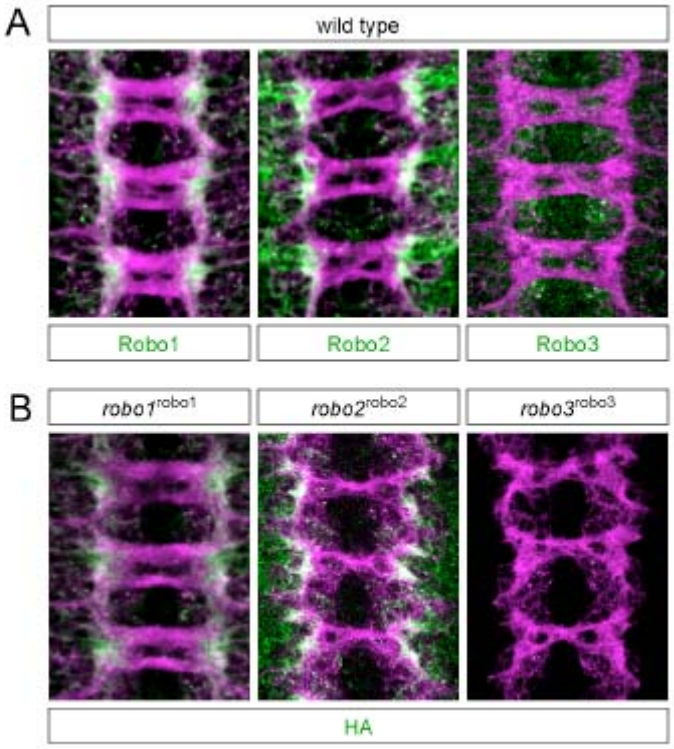

A
wild type
Robo1
Robo2
Robo3
B
robo1roba1
robo2roba2
robo3roba3
HA

Figure S1

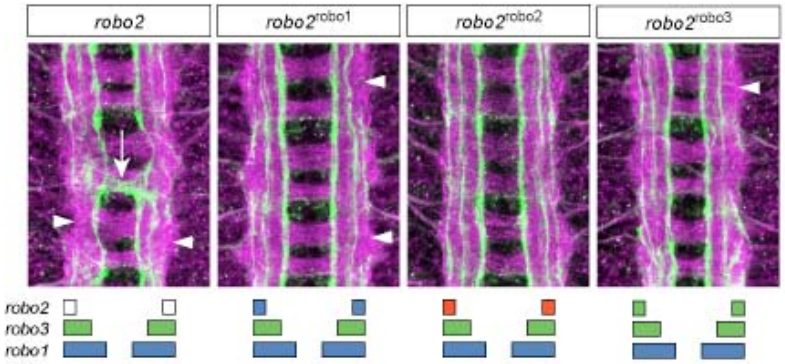


Figure S2

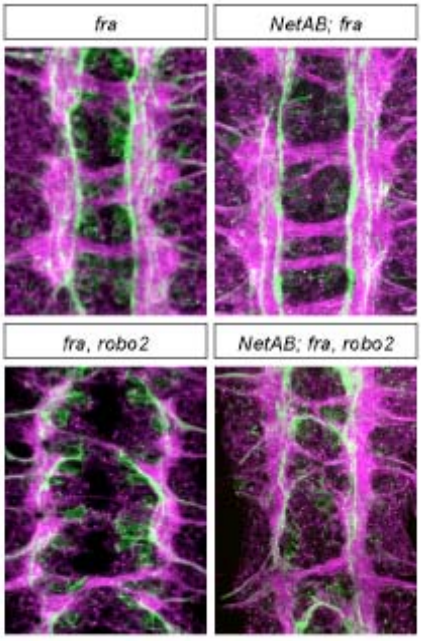


Figure S3

Chapter 2

Fine mapping of the functional specializations of the Robo receptors

Introduction

The first chapter addressed the coarse functions of the *Drosophila* Robo receptors in midline crossing and lateral positioning. We will keep the nomenclature of the Robo receptors like in the first chapter with referring to Robo as Robo1 and using Robo as a general name for the gene-family. This second chapter will deal with experiments which try to map the functions of the Robo receptors to a finer extent. First, we will discuss the results of chimeric Robo Swaps which were generated in order to assign the distinct functions of Robo1 and Robo2 to either the extra- or the intra-cellular domains. Second, we attempted to ask whether the diversity of the three Robo receptors is needed in the *Drosophila* embryonic CNS. In other words: to which extent do the distinct Robo receptors participate in midline crossing and lateral positioning when expressed as the only Robo receptor in a fly. Therefore, we generated flies expressing either Robo2 or Robo3 from all three *robo* loci. Unfortunately, we did not manage to obtain a fly expressing only Robo1 in all three loci. Third, we asked whether Robo receptors need to interact with each other in order to be functionally active. It is known that Robo1 and Robo2 can form heterodimers (Simpson, Bland et al. 2000[a;] Hivert, Liu et al. 2002) but it is not clear if these have any functions in vivo. It might be that our knock-ins, especially the ones in the *robo1* locus are not functional only because there is no such heterodimer formation. Thus we swapped the expression patterns of both, Robo1 and Robo2 as well as of both, Robo1 and Robo3. This generated flies which have both receptors but with reversed expression patterns. In a last experiment we simply addressed if all Robo Swaps of the *robo1* and *robo2* loci are fully functionally in their repulsive activity.

Results and Discussion

Chimeric Robo Swaps

In order to map the midline crossing and the lateral positioning functions to either the intra- or the extracellular domains of Robo1 and Robo2, we generated chimeric receptors

expressed from both the *robo1* and the *robo2* locus. Two different chimeric constructs were generated: either the extracellular domain of Robo1 was fused to the intracellular domain of Robo2 or vice versa (Figure 1). Planning to use these four homologous recombinant flies we wanted to assess the ability of rescuing the respective mutant phenotypes of *robo1* and *robo2*. Depending on where in one receptor or even in both receptors a given function would be located one or both of the chimeras should rescue.

Unfortunately, we did not manage to obtain all four homologous recombinant flies expressing the chimeric receptors. It was not possible to resolve the gene-duplication in the *robo1*$^{robo2\text{-}robo1}$ chimera. Therefore, our results for the *robo1* locus are incomplete and we can only analyze the *robo1*$^{robo1\text{-}robo2}$ chimeric Swap. Expressing the Robo1-Robo2 chimera in the *robo1* locus revealed an insufficient rescue of the *robo1* mutant phenotype. This fly displays a *robo1* phenotype in 64% of all commissures (Table 1 and Figure 2) indicating that some, but not all of the midline-crossing function is encoded in the extracellular domain of Robo1. It might as well be that we generated a hypomorphic Robo receptor. This might have happened by either impairing Robo1`s function through the cloning strategy or by a weaker repulsive activity of the now intracellular fused Robo2. Like this, Robo1 would recognize the correct extracellular partner(s) but would not convey a repulsive signal which is strong enough to repel all axons from the midline. However, since we miss the second chimera of the *robo1* locus, we can not sufficiently map the function of midline crossing for Robo1 or Robo2 in the *robo1* locus.

The results of the *robo2* locus are far more significant. We managed to resolve the duplication of both chimeras and first quantified their phenotypes in the wildtype background. After these results we went on and also checked the phenotypes in the *robo1* mutant and the *NetAB*$^{\Delta}$ double mutant background. First we analyzed the chimeras per se for midline crossing defects. In *robo2* mutants about 25% of the commissures have FasII positive axons crossing the midline abnormally (Rajagopalan, Nicolas et al. 2000[b;] Simpson, Kidd et al. 2000[b]). We expected both chimeras to rescue the crossing defects since already expressing the complete Robo1 protein in the *robo2* locus rescued this mild phenotype (*robo2*^{robo1} in Figure S2 of chapter 1). Thus, both parts of Robo1 – the extracellular and the intracellular – can function in midline crossing in the *robo2* locus. Indeed, both chimeric receptors did not reveal any abnormal FasII positive fascicles (Table 1 and Figure 2), confirming the result of *robo2*^{robo1}: Therefore, the repulsive contribution of Robo2 in midline crossing is substitutable by all domains of the Robo1 receptor. Next, we turned to the lateral positioning function in the wildtype background. In a robo2 mutant about 25% of the lateral fascicles stall and stop growing or shift closer

towards the midline into the intermediate fascicle (Rajagopalan, Vivancos et al. 2000[a]; Simpson, Bland et al. 2000[a]). In the *robo2*^{robo1} knock-in, midline crossing is rescued but lateral positioning phenotypes still persist (Figure S2 of chapter 1). This indicates a special lateral positioning function for Robo2 – at least for a small subset of the lateral fascicle. Thus, we wondered if this special Robo2 function relies upon the extra- or the intracellular domains. And in fact, there is a difference in the phenotypes between the two chimeric knock-ins of the *robo2* locus. The *robo2*$^{robo2\text{-}robo1}$ chimera can not rescue the *robo2* mutant lateral positioning phenotype and this chimeric Swap still displays mistakes in about 20% of the lateral fascicles (Table 1 and Figure2). In contrast to the rescuing failure of *robo2*$^{robo2\text{-}robo1}$, the *robo2*$^{robo1\text{-}robo2}$ chimera shows mistakes in only 6% of the lateral fascicles (Table 1 and Figure 2) and this hints, that there is a real qualitative different lateral positioning function of Robo2 which is located in the intracellular domain. One possible explanation might be the fourth fascicle of the Robo-code which was found in EM-studies (see also Discussion). This fascicle is located most lateral and expresses high Robo2, but no Robo3 (Simpson, Bland et al. 2000[a]). This oddity of expression might explain why there is a certain population of neurons which does not follow the level-based mechanism of lateral positioning. It simply does not follow the quantitative rules of expression of lateral positioning per se, offering Robo2 to develop another mechanism which is only revealed in this fourth Robo3 negative fascicle.

In order to prove that both chimeras are functional repulsive receptors, we crossed them into the *robo1* mutant background. A *robo1 robo2* double mutant can not repel axons from the midline since it is depleted of all early expressed repulsive receptors. Thus all axons collapse onto the midline. This is the same phenotype as in *slit* mutants where the repulsive ligand is removed (Rajagopalan, Nicolas et al. 2000[b]; Simpson, Kidd et al. 2000[b]). Single mutants for *robo1* and *robo2* prove that either one of the receptors on its own can push axons away from the midline, revealing the so-called anit-linger function of Robo1 and Robo2 (Seeger, Tear et al. 1993; Kidd, Brose et al. 1998; Rajagopalan, Nicolas et al. 2000[b]; Simpson, Kidd et al. 2000[b]). If the chimeric receptors are functional repulsive receptors they also should be able to push axons away from the midline on their own – meaning in the *robo1* mutant background, since they are inserted into the *robo2* locus. If they would be inactive they would fail in this “activity-test”, resulting in a slit-phenotype. Both chimeras seem to be functional since they reveal a *robo1*-like phenotype when recombined with the *robo1* mutant (Table 3 and Figure 3A and B). This proves that the chimeric receptors expressed from the *robo2* locus are functionally active repulsive receptors.

Next we tried to map the positive midline crossing function of Robo2 to either extra- or intracellular domains. In order to do this, we crossed the chimeras expressed from the *robo2* locus into the *NetAB*$^{\Delta}$ double mutant background. Following the same logic as in the experiments of chapter 1, one or both of the chimeras should rescue the triple mutant phenotype of *NetAB*$^{\Delta}$ *robo2*, which is completely commissureless (Figure 5 in chapter 1). In the *NetAB*$^{\Delta}$ *robo2* triple mutants 1.6% wildtype commissures form (Table 2 in chapter 1) and for a rescue we expected a significant higher number of wildtype commissures. The maximum amount of wildtype commissures we could expect would be the same amount as in *NetAB*$^{\Delta}$ double mutants: 80.4% (Table 2 in chapter 1). The *NetAB*$^{\Delta}$ *robo2*$^{robo1\text{-}robo2}$ chimera showed no rescue at all revealing only 1.6% of wildtype commissures (Table 2 and Figure 3C). In *NetAB*$^{\Delta}$ *robo2*$^{robo2\text{-}robo1}$ a much higher amount of wildtype commissures is detectable: 27.2% (Table 2 and Figure 3D), hinting that the positive function of Robo2 is located in the extracellular part of the receptor. One could imagine, that Robo2 binds to Robo1 and inhibits Slit-binding to Robo1. Hence, Robo1 would be hindered in signalling repulsion from the midline. Considering that the chimeras might be slightly compromised in their functions and considering the very delicate mechanism of midline crossing with its need for a well-balanced quantitative and qualitative interplay of partners, it might not be too surprising that the rescue is not complete. Another possible explanation for the partial rescue is an involvement of the cytoplasmic domains of Robo2 in this process.

Flies expressing only one Robo receptor in each of the three *robo* loci

A beautiful way to address to what extent one particular Robo receptor can contribute to either process - midline crossing and lateral positioning - would be to generate flies which have only one of the three Robo receptors expressed in all three *robo* loci. In the Hetero-Swaps, two instead of three distinct receptors are expressed. But we still can not exclude that the observed phenotype is reflecting the interactions between these two receptors or the functions of one receptor on its own. Thus, we generated a Robo2-only and a Robo3-only fly which has only one Robo receptor in all three loci. Analysing these flies, we can exclude any interactions between two distinct receptors and compare these flies to the Hetero-Swaps. In order to obtain these flies, we recombined two homologous recombinants. For a fly expressing Robo2 in all three loci we recombined *robo1*^{robo2} and *robo3*^{robo2}. For the Robo3-only fly we recombined *robo1*^{robo3} and *robo2*^{robo3}. Unfortunately, we did not manage to generate a Robo1-only fly since the two loci of *robo2* and *robo3* are very close together and recombining did not result in any positive event. The phenotypes

of the Robo2-only and Robo3-only fly do simply confirm the results of the Hetero-Swaps but do not add any new data (Table 1 and Figure 4). Both flies show only the mere combination of the two recombined phenotypes. The CNS of the Robo2-only fly (Figure 4A) has the same defects in midline crossing as the *robo1* mutant or the knock-in of *robo1*^{robo2}. The *robo3* mutant lateral positioning phenotype of the intermediate fascicle is rescued in the Robo2-only fly as it is in the knock-in of *robo3*^{robo2}. The phenotypes of the Robo3-only fly (Figure 4B) too, resemble the two recombined knock-ins. *robo1`s* midline crossing phenotype is not rescued as it is in *robo1*$^{robo3}$ and also *robo2`s* lateral positioning phenotype is not rescued as in the knock-in of *robo2*^{robo3}. Thus, this shows that midline repulsion is controlled by Robo1 and lateral positioning is done by all Robo receptors.

Swapping the expression patterns of two Robo receptors at the same time

One concern of the Robo Swaps was that a knocked-in receptor might not be able to act its part because it does not have the right partner to do so. The *robo1*^{robo2} knock-in fly has two distinct Robo2 receptor copies produced by the two different loci – *robo1* and *robo2* - but it does not express any Robo1. In line with the above argument, the *robo1*^{robo2} knock-in would not rescue because Robo2 needs Robo1 for its midline crossing function. Therefore, we recombined the *robo1*^{robo2} knock-in with the *robo2*^{robo1} knock-in thereby creating a fly which bears the two different receptors but in the reversed expression patterns. Again, the phenotype of this fly displays only the mere combination of the two recombined knock-ins (Table 1 and Figure 5). We see lateral positioning mistakes in the lateral fascicle reflecting the failure of Robo1 to replace Robo2 in lateral positioning. Additionally, Robo2 can not replace Robo1 in midline crossing, resulting in a *robo1* phenotype (Table 1 and Figure 5). Hence, in the wild-type background Robo2 can not replace Robo1`s midline repelling function even though Robo1 is provided from a different locus. This result confirms the trans-heterozygous experiments of chapter 1. There we asked whether Robo1 could rescue midline crossing when expressed from a different locus. But, *robo1*^{GA285} *robo2*^{robo1}/ *robo1*^{GA285} and *robo1*^{GA285} *robo3*^{robo1} / *robo1*^{GA285} could rescue the *robo1* mutant phenotype (Figure 4 in chapter 1). We also recombined *robo1*^{robo3} with *robo3*^{robo1} thus swapping Robo1 and Robo3 completely. This fly also confirmed the so far obtained results. We discovered a *robo1* phenotype (Table 1- Figure not shown) which is explained by the failure of *robo1*^{robo3} to rescue midline crossing of the *robo1* mutant and the result, that Robo1 can not rescue midline crossing when expressed in the *robo3* locus (Figure 4 in chapter 1).

Addressing the repulsive activity of the Robo Swaps

Taking into consideration, that the Iso- Swaps rescue completely their respective mutant phenotypes (Table1 in chapter 1) and the resulting phenotypes of all Robo-Swaps are highly reproducible in all tested genetic backgrounds, it is quite unlikely that the Robo Swaps are compromised in their functions. But we have a way to confirm the repulsive functionality of all Robo Swaps. We followed the same logic as in the article about the chimeric Robo Swaps in chapter 2. In a *robo1 robo2* double mutant all axons collapse onto the midline and are not able to leave it, the single mutants on the other hand reveal the repulsive function of Robo1 and Robo2 respectively (Kidd, Brose et al. 1998; Rajagopalan, Nicolas et al. 2000[b;] Simpson, Kidd et al. 2000[b]). Thus, we recombined all Robo Swaps of the *robo1* locus into the *robo2* mutant background and all Robo Swaps of the *robo2* locus into the *robo1* mutant background. Then we asked whether the Swaps can push the axons away from the midline in the absence of any other early repulsive receptor, or not. We did not test the Swaps of the *robo3* locus since already $robo3^{robo1}$ can not rescue midline crossing. Table 2 summarizes the results and shows, that all Robo Swaps kept the repulsive functionality to more or less the same extent since they reveal rather a *robo1* than a *slit* mutant phenotype.

Conclusions

Finally, we would like to conclude from our chimeric studies that the repulsive midline crossing contribution of Robo2 is substitutable by all parts of the Robo1 receptor repeating the result of the $robo2^{robo1}$ knock-in. Second, there seems to be a Robo2 specific lateral positioning function – at least for a small subset of the lateral fascicle - which can not be explained through a level based mechanism and it seems to be located in the intracellular domain of Robo2. Third, it is the extracellular domain which seems to be responsible for the positive contribution to Robo2`s midline crossing promoting function. Our experiments which swapped the expression patterns of two Robo receptors at the same time confirmed that Robo1 can not be replaced by Robo2 or Robo3 – even though all three Robo receptors are present at the growth-cone. Thus, Robo2 and Robo3 do not lack this midline repelling function of Robo1 because Robo1 can not interact with them on the growth-cone, but they do not encode this function in their protein domains. As a last experiment we excluded that the Robo Swaps are compromised in their repulsive functionality showing that they kept their anti-linger function at the midline.

Material and methods

Generation of chimeric Robo Swap Alleles

Each of the four chimeric *robo* swap alleles was generated by ends-in homologous recombination (Rong and Golic 2000). Four donor constructs were prepared in P-element vectors, each containing a single exon encoding the desired HA-tagged chimeric Robo protein in the correct reading frame for fusion with the signal sequence encoded in the first exon of either the *rob-o* or the *robo2* gene. This replacement exon was flanked on the 5' side by 7.5–7.7 kb of genomic DNA from the locus to be targeted, and 1.4–1.9kb on the 3' side. An I-SceI site was included roughly in the middle of the 5' homology region. Two I-CreI sites at the distal end of the 3' homology region are separated by a mini-*white* marker. The entire targeting cassette was flanked by FRT sites. These constructs were prepared using standard PCR-based cloning procedures, using genomic DNA from the w^{1118} strain and plasmids containing the *robo* or *robo2* cDNAs as templates. All coding regions and cloning junctions were confirmed by DNA sequencing. Transgene insertions on the X or 3rd chromosome were used for targeting, as all three *robo* genes are located on the 2nd chromosome.

The targeting fragment was then liberated and linearized in the female germline using FLP and I-SceI, respectively, and progeny were screened for movement of the mini-*white* marker to the 2nd chromosome, as well as its resistance to *ey*FLP (indicating that it is no longer flanked by FRT sites, as in the donor; (Newsome, Asling et al. 2000). The successful generation of 1–6 homologous recombinants per allele was initially confirmed using a set of specific PCRs to detect the insertion of the replacement *robo* sequence and the disruption of the endogenous locus. The initial recombinants contained a duplication at the intended locus, which was subsequently resolved by using I-CreI to induce a double-stranded break and selecting in the progeny for the loss of the intervening $white^{+}$ marker. These recombinants were then screened by PCR to identify those that retained the replacement allele and had lost the endogenous allele, prior to the more extensive histological characterisation as described in the Results.

Immunohistochemistry

Immunofluorescence stainings of staged and fixed embryos were performed as described (Patel 1994). Primary antibodies used were anti-FasII mAb 1D4 (1:1000, (Vactor, Sink et

al. 1993), anti-ß-galactosidase (1:1000, Promega) and Cy5-conjugated sheep anti-HRP (1:500, Jackson Immunoresearch). Secondary antibodies used were anti-mouse Alexa Fluor-488 and anti-rabbit Alexa Fluor-568-conjugated (1: 1000, Molecular Probes). Homozygous embryos were identified by selecting against anti-ß-galactosidase staining indicating the presence of CyO, P[*wg-lacZ*] the balancer chromosome. Selected embryos were dissected, mounted in Vectashield mounting medium (Vector Labs) and images were acquired with a Zeiss LSM 510 confocal microscope.

Quantification of Midline Crossing and Lateral Positioning defects

For the quantification of defects in the commissural or longitudinal axon pathways, stage 16 or 17 embryos were stained with mAb BP102 or anti-FasII, respectively, using the Vectastain Elite ABC kit. Anti-ß-galactosidase was included to allow identification of the mutant embryos, which were dissected and mounted in 70% glycerol. Phenotypes were scored blind to the genotype using Nomarski optics on a Zeiss Axioplan 2 microscope with a 100 X objective.

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Figure legends

Table 1. Chimeric Robo receptor Swaps, complete Swaps of Robo receptors and flies expressing only Robo2 or only Robo3

Stage 16-17 embryos stained with anti-FasII were scored for the presence of FasII-positive axons extending across or along the midline, and for breaks in the intermediate and lateral FasII fascicle, often due to fusion with the more medial fascicle. Indicated are the genotype, number of hemisegments scored, percentage of segments with FasII positive axons at the midline and percentage of mistakes in lateral positioning each for the medial (m), the intermediate (i) and the lateral (l) fascicle.

Table 2. Commissure Formation in the chimeric Robo Swaps combined with the *NetAB*$^{\Delta}$ double mutants.

Stage 16-17 embryos stained with BP102 were scored for defects in the anterior and posterior commissures.

Table 3. Estimation of the degree of neuropil formation and fasciculation in different mutants and Robo Swaps.

Stage 16-17 embryos stained with anti-FasII were scored for the formation of a wildtype CNS (three distinct FasII fascicles, normal width of the neuropil), a *robo1* phenotype (abundant crossing medial fascicle, condensed neuropil) or a *slit* phenotype (collapse of all fascicles on the midline, very condensed neuropil).

Figure 1. Schematic of Chimeric Robo receptor Swap proteins.

Schematic of the protein domains of the two constructs for the chimeric Robo Swaps. Both constructs are expressed in either the robo or the *robo2* locus. A) The extracellular region is comprised of Robo domains (blue) and the intracellular domains are made up of the Robo2 protein (red). B) Extracellular Robo2 (red) and intracellular Robo (blue). Ig: Immunoglobulin- domains; FN: Fibronectin III- domains; TM: transmembrane domain; cc0-cc3: conserved consensus sequences.

Figure 2. Visualization of the phenotypes of the chimeric Robo Swaps.

Stage 16-17 embryos of the indicated genotype, stained with anti-FasII (green) and anti-HRP (magenta). A) *robo1*$^{robo1\text{-}robo2}$ shows a *robo1* mutant phenotype. B) *robo2*$^{robo1\text{-}robo2}$ looks very much like wildtype embryos and *robo2*$^{rob2o\text{-}robo1}$ resembles more a *robo2* mutant (C).

Figure 3. Visualization of the phenotypes of the chimeric Robo Swaps in different genetic backgrounds.

Stage 16-17 embryos of the indicated genotype, stained with anti-FasII (green) and anti-HRP (magenta). A) *robo1 robo2*$^{robo1\text{-}robo2}$ shows a *robo1* mutant phenotype. B) *robo1 robo2*$^{robo1\text{-}robo2}$ looks more condensed but still *robo1* like. In a *NetAB*$^{\Delta}$ *robo2*$^{robo1\text{-}robo2}$ (C) all commissures are thinned and look only in 1.6% of the cases like wildtype. A *NetAB*$^{\Delta}$ *robo2*$^{robo1\text{-}robo2}$ fly has more wildtype commissures even though some commissures are missing.

Tables

Genotype	Hemi-segment	FasII positive axons at midline	Mistake of FasII fascicles (%)					
			m+i	i+l	m+i+l	Break l	Break i	Break m
wild type	610	0	0	0	0	0	0	0
robo1^{GA285} / *robo1*^{Z3127}	288	100.0*	0	0.7	0	0.7	0	0
Robo1$^{robo1\text{-}robo2}$	314	63.7*	0	0.96	0	0	0	0
robo2^{4}	186	23.7	10.2	19.9	0	10	1.1	2.2
robo2$^{robo1\text{-}robo2}$	250	8	2	4.4	0	4	0	0
robo2$^{robo2\text{-}robo1}$	350	0.57	0	12.9	0	6	0	0
robo^{robo2},*robo2*^{robo1}	254	100.0	0	13.5	0	7	1.2	0
robo1^{robo3},*robo3*^{robo1}	232	78**	-	-	-	-	-	-
robo1^{robo2},*robo3*^{robo2}	332	98.8	0.9	0.9	0	0.3	0	0
robo1^{robo3},*robo2*^{robo3}	240	100**	-	-	-	-	-	-

Table 1

* *robo1*-like phenotype

** *slit*-like phenotype

Genotype	Hemi-segments	Anterior Commissure			Posterior Commissure		
		normal	thin	absent	normal	thin	absent
wild type	437	100	0	0	100	0	0
NetAB$^{\Delta}$	276	80.4	13.8	5.8	55.8	26.8	17.4
NetAB$^{\Delta}$*; robo2*^{4}	247	1.6	27.6	70.7	0	12.9	87.1
NetAB$^{\Delta}$*; robo2*$^{robo1\text{-}robo2}$	436	4.5	57	38.6	0.5	25.8	73.7
NetAB$^{\Delta}$*; robo2*$^{robo2\text{-}robo1}$	162	27.2	30.9	42	22.2	21	56.8

Table 2

Genotype	Segments scored	*robo1* phenotype (%)	Slit phenotype (%)	Wildtype (%)
wild type	305	0	0	100
robo1^{GA285}*robo2*^{4}	154	81.8	18.2	0
robo1^{GA285} / *robo1*^{Z3127}	288	100.0	0	0
robo1 ^{GA285} *robo2*^{robo1}	332	48.8	16.7	34.9
robo1 ^{GA285} *robo2*^{robo2}	497	100.0	0	0
robo1 ^{GA285} *robo2*^{robo3}	360	34	76	0
robo2^{4}/ *robo2*^{8}	186	0	0	76.3*
robo2^{4} *robo1*^{robo1}	240	100	0	0
robo2^{4} *robo1*^{robo2}	400	100	0	0
robo2^{4} *robo1*^{robo3}	242	100	0	0
robo1, robo2$^{robo1\text{-}robo2}$	396	81.8	10.1	8.1
robo1, robo2$^{robo2\text{-}robo1}$	240	100	0	0

*The *robo2* mutants have in 23.7% of the segments midline crossing defects.

Table 3

Figures

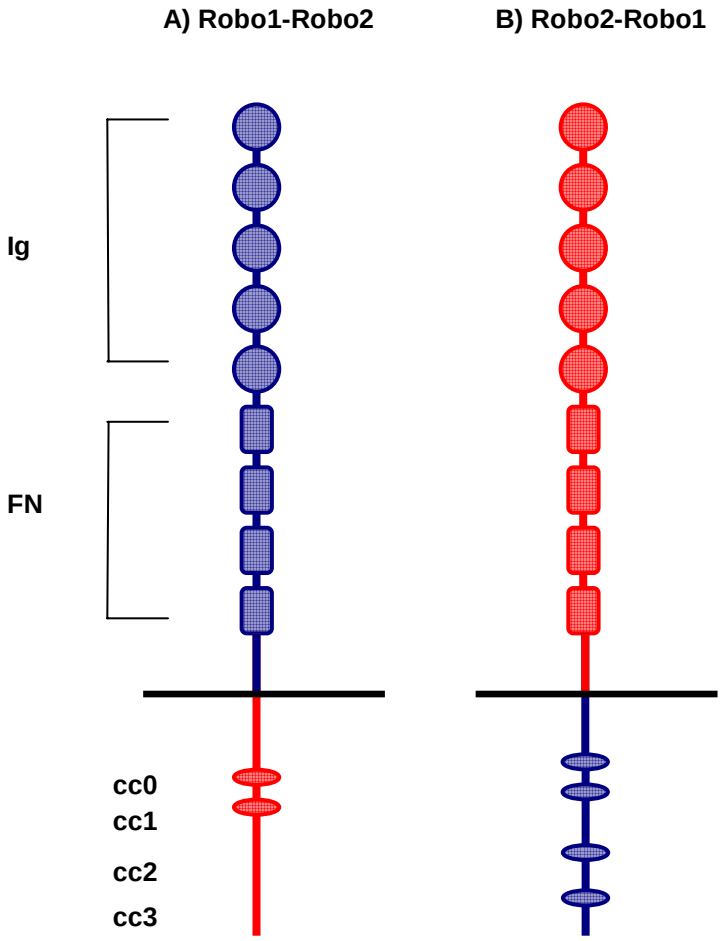

Figure 1

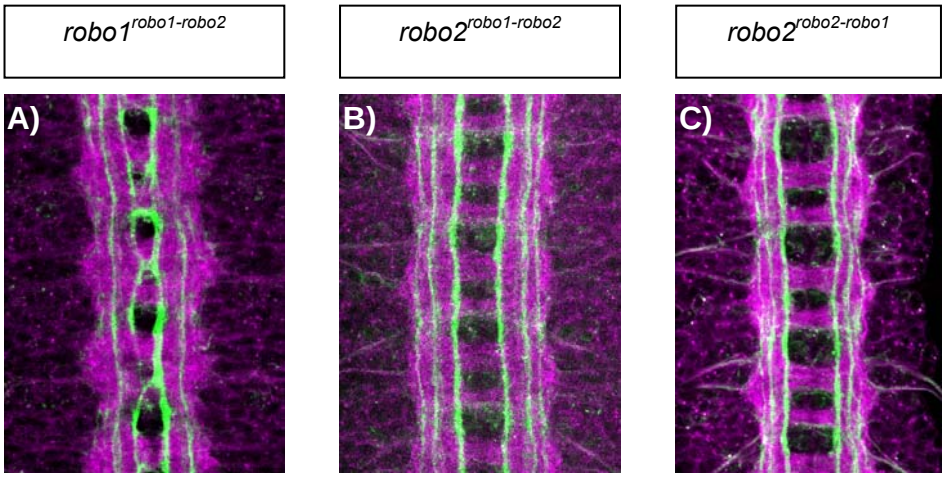

Figure 2

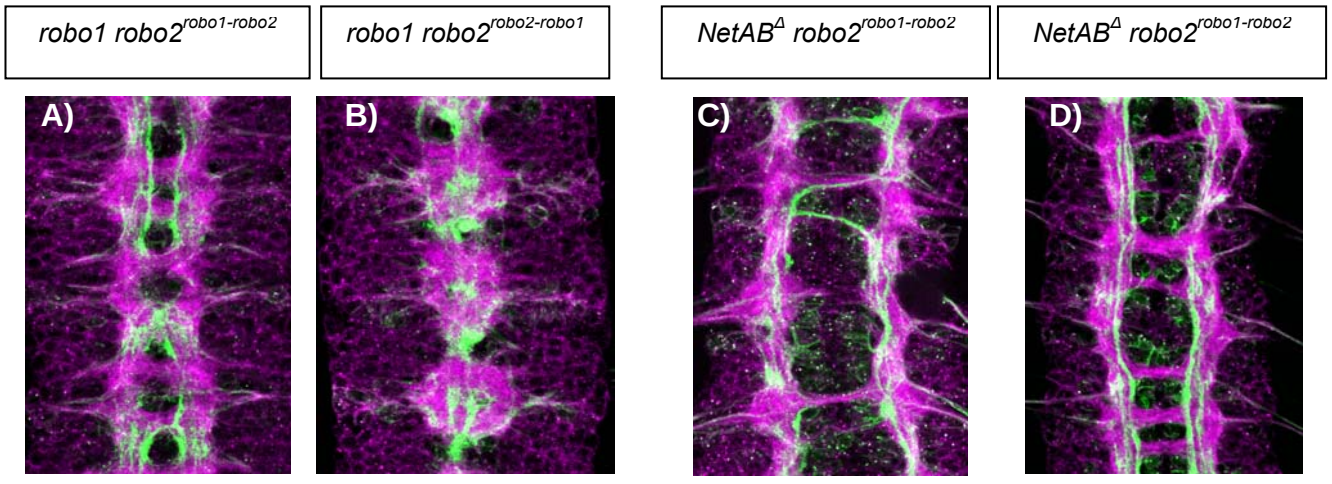

Figure 3

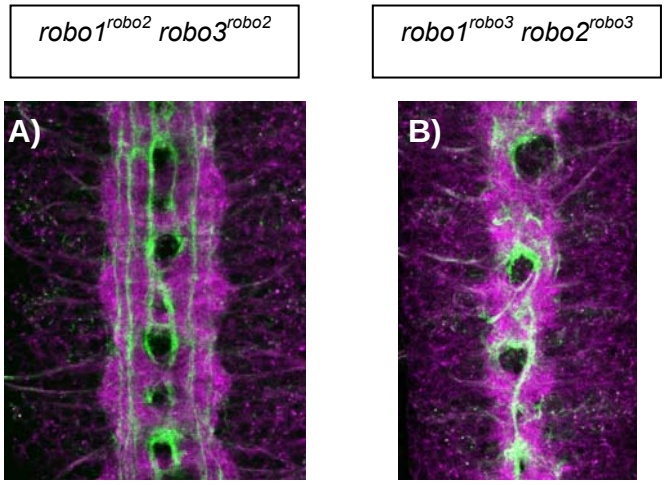

Figure 4

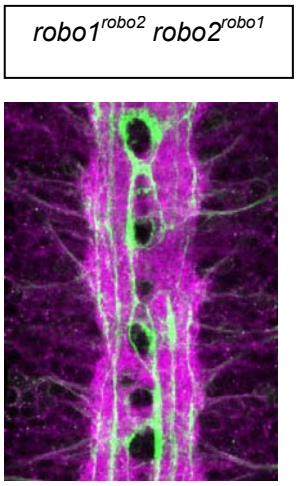

Figure 5

Discussion

In *Drosophila* three Robo receptors help form the orthogonal array of the embryonic CNS. Slit is their common repulsive ligand and the Robo receptors are involved in two processes. The one process decides about crossing the midline of the embryo or to stay on the ipsilateral side. The other process is about which lateral pathway to choose. The functions in these two processes are distinct for the different Robo receptors. Robo1 controls repulsion from the midline through being or not being expressed on the growth-cone (Kidd, Brose et al. 1998; Kidd, Russell et al. 1998; Keleman, Rajagopalan et al. 2002) and this function is unique for Robo1. Robo2 seems to have only a mild contribution to this repulsive process (Rajagopalan, Vivancos et al. 2000[a;] Simpson, Bland et al. 2000[a;] Rajagopalan, Nicolas et al. 2000[b;] Simpson, Kidd et al. 2000[b]) and seems to enable crossing of axons, a newly discovered function. Robo3 also seems to contribute little to midline repulsion (Rajagopalan, Vivancos et al. 2000[a;] Simpson, Bland et al. 2000[a;] Rajagopalan, Nicolas et al. 2000[b;] Simpson, Kidd et al. 2000[b]) and has a major role in lateral pathway selection (Rajagopalan, Vivancos et al. 2000[a;] Simpson, Bland et al. 2000[a]). This role is not specific to Robo3, but encoded in all three Robo receptors. These distinct functions may reflect one of the two differences between the Robo receptors: the distinct features of their protein-domains or the different spatial and temporal expression patterns (Rajagopalan, Vivancos et al. 2000[a;] Simpson, Bland et al. 2000[a;] Rajagopalan, Nicolas et al. 2000[b;] Simpson, Kidd et al. 2000[b]).

The aim of this thesis was to determine where the distinct functions of the Robo receptors are encoded either in the features of the protein-domains (and in which of these domains exactly) or in their expression patterns. In order to address this we asked whether the Robo receptors are replaceable with each other. If one receptor can functionally substitute for the other they have this same function encoded in their protein domains and the difference between the two receptors reflects their distinct expression patterns. If a receptor can not be replaced by another, they have different functions within their protein domains. Thus, we generated nine homologous recombinant fly-strains in which we swapped the Robo receptors. We substituted each Robo receptor with itself (iso Swaps) and the other two Robo receptors (Hetero Swaps). First we confirmed that the expression patterns of the knocked-in receptors (Iso Swaps and Hetero Swaps) were unaltered. Then we checked that the controls (Iso Swaps) did not show any phenotype before we analyzed the phenotypes of the Hetero Swaps asking if they can replace one another in the different processes: midline crossing and lateral pathway selection.

Lateral positioning follows a level dependent mechanism

Different hypotheses have been proposed to explain lateral pathway selection. One of the first models was the “labelled pathway hypothesis” (Raper, Bastiani et al. 1983; Goodman, Bastiani et al. 1984; Raper, Bastiani et al. 1984). Experiments in grasshoppers, including the mere observation of fascicles and single cells, labelling- and cell ablation experiments showed that certain cells always select the same pathways. This finding together with the discovery of the first pathway labels (Bastiani, Harrelson et al. 1987; Grenningloh, Rehm et al. 1991; Grenningloh and Goodman 1992; Kolodkin, Matthes et al. 1993) led to the idea that each pathway is designated by a specific subset of markers guiding a neuron unambiguously to its target. However, it became obvious that there are not enough markers to cover the enormous amount of alternative pathways (Lin, Fetter et al. 1994). After the discovery of the Robo receptor family, their expression patterns, mutant- and misexpression phenotypes, a new idea of how axons choose their pathways was born: Robo receptors sort axons into broad regions and pathway labels specify the more precise choices within one of these regions (Rajagopalan, Vivancos et al. 2000[a;] Simpson, Bland et al. 2000[a]). Robo receptors in late stages show distinct and overlapping zones of expression generating axons with Robo1 only in a medial pathway, an intermediate pathway with Robo1 and Robo3 expressing axons and a lateral pathway with axons bearing all three Robo receptors (Rajagopalan, Vivancos et al. 2000[a;] Simpson, Bland et al. 2000[a]) (Figure 10 in introduction). But how exactly do the Robo receptors instruct axons about their lateral pathways? One experiment lead to two different proposed models. The setup was a series of misexpression experiments using the UAS-Gal4 system in ipsilateral single-axon-markers which normally run medial and therefore express only Robo1 (Apterous-neurons). Misexpressing Robo1 in these medial neurons did not shift the axons further lateral but misexpression of Robo2 or Robo3 did lead to shifts to different extents (Rajagopalan, Vivancos et al. 2000[a;] Simpson, Bland et al. 2000[a]). The observation that misexpressing Robo3 shifts axons into the intermediate fascicle and misexpression of Robo2 shifts them even further lateral into the lateral fascicle spiked the idea that there are qualitative different signals between the two receptors and each of them leads the axons into a distinct pathway (Simpson, Bland et al. 2000[a]). In this model it would depend on the specific features of the protein-domains in each receptor, which fascicle is chosen. Robo1 would specify the medial fascicle through absence of Robo2 and/ or Robo3, Robo3 would specify the intermediate and Robo2 the lateral fascicle. Another interpretation of the Ap-experiments was that overexpression of either Robo2 or Robo3 on its own leads axons into the same intermediate fascicle and only higher amounts of combined receptor-amounts (1x Robo2 and 1x Robo3 or 2x

Robo3) leads axons into the lateral fascicle giving rise to the idea of a level-dependent model where total amounts of any of the two receptors define lateral positions (Rajagopalan, Vivancos et al. 2000[a]). Like this, axons with no or low levels – like the ones with only Robo1 would stay medial, axons with mediocre levels – like Robo1 and Robo3 expressing axons - would go intermediate and axons with high levels provided through all three Robo receptors would move furthest away from the midline into the lateral fascicle. Most probably this level-dependent model reflects differences in space and differences in time. At least for *robo3* it is clear that it is turned on later and thus provides additional Robo3 around S14/15 (Rajagopalan, Vivancos et al. 2000[a;] Simpson, Kidd et al. 2000[b])

The definitive experiment to distinguish between these two hypotheses is to ask whether the Robo receptors are substitutable. The qualitative model would predict shifts of the fascicles according to which receptor has been placed onto the growth cone. All axons with only Robo would stay in the medial fascicle, axons expressing any amount of Robo3 would go into the intermediate and all axons with any Robo2 into the lateral fascicle. For example, misexpressing Robo2 in intermediate axons should shift them into the lateral zone (Figure 1 B) since their expression pattern changed from Robo1-Robo3 to Robo1-Robo3-Robo2. In the level dependent model on the other hand it should not matter which of the Robo receptors is expressed where, it is only the combined amount of all of them which matters. In this quantitative model, all hetero Swaps should not reveal any lateral positioning defects - visualized in the example of *robo3*^{robo2} - since all Robos can substitute for each other.

The results of this study suggest a model in which expression patterns specify lateral positions. Especially for the *robo3* locus evidence is prominent. In a *robo3* mutant, the intermediate fascicle moves closer to the midline and merges completely with the medial fascicle (Rajagopalan, Vivancos et al. 2000[a;] Simpson, Bland et al. 2000[a]). The knock-ins of the Robo1 and the Robo2 receptor into the *robo3* locus (*robo3*^{robo1} and *robo3*^{robo2}) are able to restore the outward shift of the intermediate fascicle (Figure 2 and Table 1 in chapter 1). This shows that Robo3 is completely replaceable with Robo1 and Robo2 and thus they all have the same lateral positioning functions within their domains. This wildtype lateral positioning result is in accordance with the level-dependent model in which combined amounts of Robo receptors define lateral pathway selection and Robo receptors are substitutable.

The results of the *robo2* locus unfortunately do not display the same clarity. Neither *robo2*^{robo1} nor *robo2*^{robo3} seem to be able to rescue the *robo2* mutant phenotype where

about 25% of the lateral fascicle shift into the intermediate fascicle or stop and stall (Rajagopalan, Vivancos et al. 2000[a]; Simpson, Bland et al. 2000[a]). Additionally, *robo2*^{robo1} can rescue the defects of the *robo2* mutant in midline crossing, but *robo2*^{robo3} can not. A first thought would be compromised functions of the hetero Swaps in the *robo2* locus in lateral positioning. But given the perfect rescue of *robo2*^{robo2} and no other reasons to believe in compromised Robo Swap functions, this is rather unlikely. We can even refute the argument that *robo2*`s lateral positioning phenotype is a mere secondary effect of the crossing defects in this mutant (Rajagopalan, Vivancos et al. 2000[a]) because *robo2*^{robo1} rescues this crossing defect but still shows the lateral positioning mistakes. Thus, we believe that there is a Robo2 function in lateral positioning which is based on qualitative differences between Robo2 and the other two Robo receptors.

However, we do not believe that the results of the knock-ins of *robo2*^{robo1} and *robo2*^{robo3} contradict the level-based model for lateral positioning. The reasoning includes the assumption that the lateral fascicle may be heterogeneous in its build-up and thus in its development. First, the lateral fascicle comprises different expression zones of Robo receptors. It consists of a major Robo1-Robo2-Robo3 positive part and a small Robo1-Robo2 expressing region as shown by EM-studies (Simpson, Bland et al. 2000[a]). Second, the *robo2* mutant does not give the same clear phenotypes as the other two *robo* mutants. In both, *robo1* mutants and *robo3* mutants, all neurons of one or more fascicles are affected. In *robo2* mutants only a further not defined subset fails to position correctly – or fails to cross the midline only once. Third, misexpression of Robo2 in Ap-neurons does not shift axons into a defined lateral position but makes them choose sometimes the intermediate and other times the lateral fascicle (Rajagopalan, Vivancos et al. 2000[a]; Simpson, Bland et al. 2000[a]). Thus, the idea is that there are two different mechanisms for the two differently equipped lateral pathways. The lateral fascicle would house a large and level dependent Robo1-Robo2-Robo3 area and a small Robo1-Robo2 region which relies on special features of the Robo2 receptor. Usually both mechanisms work together to build up the lateral fascicle. In a *robo2* mutant this balance is broken and it might be that sometimes the domain-dependent neurons grow out first and can not find their lateral position since Robo2 is missing and can not instruct the axons properly. The followers would simply grow in the misguided pathways leading to the 25% of lateral positioning defects. In most of the cases, the level-dependent neurons would lead the way and in these axons it might be possible that Robo3 or Robo1 can somehow substitute for Robo2 (maybe through the late expression pattern of Robo3). These axons can find their proper lateral target because they simply express more total Robo receptor amount than the intermediate fascicle. And again the followers would just position

correctly since they follow these pathways. This hypothesis would fit with the results of the Robo Swaps. In the *robo2*^{robo1} knock-in, Robo1 can indeed replace Robo3 in the level-dependent Robo1-Robo2-Robo3 zone and additionally, it might rescue the Robo2 function in this, but not in the Robo1-Robo2 zone where Robo2 has specific lateral positioning functions. The same explanation works for the *robo2*^{robo3} knock-in, Robo3 replaces itself and Robo2 in the level-dependent zone but it can not rescue Robo2`s specific functions in the domain-dependent zone. Therefore, depending on which neurons pioneer the pathway the lateral fascicle forms correctly or fails to form, as it happens in the *robo2* mutant.

In order to map the specific lateral positioning function of Robo2 to either the intra- or extracellular domains we used chimeric Robo receptor knock-ins of the *robo2* locus. Only the *robo2*$^{robo1\text{-}robo2}$ chimera with intracellular Robo2 domains, but not the *robo2*$^{robo2\text{-}robo1}$ chimera with extracellular Robo2 can partially rescue the 25% misguided lateral fascicles of the *robo2* mutant. The *robo2*$^{robo1\text{-}robo2}$ knock-in displays only about 6% of lateral positioning mistakes whereas r*obo2*$^{robo2\text{-}robo1}$ reveals about 20% of lateral mistakes and thus does not rescue at all. A possible conclusion is that the extracellular domains specialise Robo2 for this specific domain-dependent position within the lateral fascicle.

Several other results would accord with the hypothesis that Robo2 is involved in two distinct functions in the formation of the lateral fascicle. First, the *robo2*^{robo1} knock-in and the *robo2*^{robo3} –knock-in fail to rescue only the 25% of the lateral positioning phenotype of the *robo2* mutant but do not fail to establish the major level-dependent part of the fascicle. This confirms that Robo1 and Robo3 might somehow replace Robo2 in the level-dependent area but lack the specific Robo2 function. And second, in a *robo3* mutant a thinned lateral fascicle forms. If only levels would matter for lateral positioning, this should not happen and the complete lateral fascicle would shift closer to the midline according to the lesser amounts of Robo receptors on its growth cone. A Robo2 specific lateral positioning mechanism for a subset of the lateral fascicle would explain why this thinned fascicle forms at the very edge of the neuropil. Robo2 guides axons to a certain pathway because of its specific functions. The correct formation of a thinned, but undisturbed fascicle would be explained by the fact that in *robo3* mutants the level-dependent large area of the lateral fascicle does not develop due to loss of Robo3. Therefore this level-dependent part can not disturb the formation of the domain-dependent part of the lateral fascicle.

Unfortunately, there are no specific pathway labels for the lateral fascicle available and thus we can not dissect its function using single-axon markers like they exist for the medial and the intermediate fascicle. But one more way to approach this question genetically would be to generate a fly which expresses Robo1 in all three genetic *robo* loci. Secondary effects from not passing the midline would be prevented by expressing Robo1 in the *robo1* and in the *robo2* locus. The intermediate fascicle should be rescued by expressing Robo1 in the *robo3* locus and also the assumed level-dependent major part of the lateral fascicle should be rescued in this fly since Robo levels should be wildtype. Now, if Robo2 would instruct a certain subset of the lateral fascicle specifically, this would not be rescued by Robo1 and result in a typical *robo2* mutant lateral positioning phenotype. If the level based mechanism is also true for this part of the lateral fascicle, Robo1 should be able to replace Robo2 and rescue the lateral fascicle completely. Why do we think it makes a difference to the $robo2^{robo1}$ hetero Swap which does not rescue this part of the fascicle? In the $robo2^{robo1}$ fly Robo3 is still expressed in the intermediate and most of the lateral fascicle. This may interfere somehow with the formation of the Robo2-dependent special part of the lateral fascicle. Maybe Robo2 needs Robo3 for its special function? Maybe Robo3 hinders Robo1 in $robo2^{robo1}$ to form the last bit of the lateral fascicle? However, in the Robo1-only fly there is no Robo3 expressed and we can exclude this possible interference.

Concluding, we would like to state that the main mechanism for lateral positioning is following a level-based mode. The more of any Robo receptor an axon expresses the further away it is repelled from the midline. We refute a combinatorial code for lateral positioning for the intermediate fascicle and assume that most of the lateral fascicle also follows quantitative levels. There might be a unique lateral positioning function for Robo2 which applies only for a small subset of lateral neurons and it might be encoded in the intracellular domains of Robo2.

Midline repulsion is controlled uniquely by Robo1

The *Drosophila* embryo has a bilateral symmetric CNS with a central midline which is crossed by ~ 80- 90% of the neurons. The crossing is facilitated by the redundant Netrins which are expressed from the midline and their ubiquitous receptor Frazzled (Harris, Sabatelli et al. 1996; Kolodziej, Timpe et al. 1996; Mitchell, Doyle et al. 1996). Inhibition of crossing is mediated by Slit, generated from midline cells (Kidd, Bland et al. 1999) and its three Robo receptors, which are present on non-crossing neurons only (Kidd, Brose et

al. 1998; Simpson, Bland et al. 2000[a;] Rajagopalan, Nicolas et al. 2000[b]). The single and double mutant phenotypes of the Robo receptors show that it is mainly Robo1 which contributes to midline repulsion. In a *robo1* mutant all axons cross and recross the midline abundantly (Kidd, Brose et al. 1998), in *robo2* mutants only one quarter fails in crossing correctly only once and in *robo3* mutants even less crossing phenotypes are visible (Rajagopalan, Nicolas et al. 2000[b;] Simpson, Kidd et al. 2000[b]). This reflects the different contributions towards midline crossing for each Robo receptor. Even more, in *robo1 robo2* double mutants only Robo3 is expressed at late stages and all axons collapse onto the midline without being able to leave it again (Rajagopalan, Vivancos et al. 2000[a;] Simpson, Bland et al. 2000[a;] Simpson, Kidd et al. 2000[b]). This is the same phenotype as in *slit* mutants where no axons can leave the attractive midline because it is depleted of all repulsive force from the ligand (Kidd, Bland et al. 1999). In *robo1 robo3* double mutants only Robo2 is expressed and thus, some axons are able to leave the midline forming a *robo1* mutant CNS plus the lateral positioning defects of the *robo3* mutant. *robo2 robo3* double mutants express Robo1 only and therefore a relative normally shaped neuropil forms with strong defects in lateral positioning (Rajagopalan, Vivancos et al. 2000[a;] Simpson, Bland et al. 2000[a;] Simpson, Kidd et al. 2000[b]). Thus, it was assumed that Robo1 is the most important molecule in midline repulsion and Robo2 and Robo3 merely ensure the fidelity of it (Rajagopalan, Nicolas et al. 2000[b;] Simpson, Kidd et al. 2000[b]).

But is this assumption really true? Do Robo2 and Robo3 indeed not contribute to midline repulsion or are these functions merely obstructed by their expression patterns? Would they be as powerful in midline repulsion as Robo1 if they were expressed as *robo1*? We used the knock-ins of the Robo2 and Robo3 proteins into the *robo1* locus to ask whether the midline repelling functions are unique for the features of the protein-domains of Robo1 or whether they can be replaced by the other two Robo receptors.

The knock-ins into the *robo1* locus show, that neither Robo2 nor Robo3 can improve the *robo1* mutant phenotype when they are expressed in the *robo1* locus. Still all the axons cross and recross the midline abundantly. Therefore, midline repulsion is mediated by the features of the protein-domains of Robo1 and neither encoded in Robo2- or in Robo3-domains. This midline repelling function might be localized in the intracellular domains cc2 and cc3, which both, Robo2 and Robo3 lack when compared to Robo1 (Rajagopalan, Nicolas et al. 2000[b;] Simpson, Kidd et al. 2000[b]).

We do not know which domains of the Robo1 receptor are special for midline repulsion. We can not exclude that the extracellular domains make the difference. But we rate it rather unlikely since all three Robo receptors have a very similar structure (Rajagopalan, Vivancos et al. 2000[a;] Simpson, Bland et al. 2000[a]) and it seems that their binding capacity for Slit is in a similar range (Simpson, Bland et al. 2000[a;] Howitt, Clout et al. 2004). Another domain which distinguishes between the three Robo receptors in midline repulsion is the transmembrane domain. It seems that only a small region around the transmembrane domain of Robo1 is sufficient for its sorting through a small transmembrane protein named Comm (Tear, Harris et al. 1996; Georgiou and Tear 2003; Gilestro 2006). In commissural neurons, Comm sorts Robo1 directly from the Golgi to the endosomal degradation machinery without allowing it to reach the cell surface (Keleman, Rajagopalan et al. 2002; Myat, Henry et al. 2002). Thus, Robo1 expression is confined to non-crossing neurons repelling them from the midline. But Comm can also sort Robo2 and Robo3. Obviously, both receptors are as well restricted in their expression to longitudinal pathways and excluded from commissures (Rajagopalan, Vivancos et al. 2000[a;] Simpson, Bland et al. 2000[a]). Another hint that Comm regulates all three Robo receptors comes from pan-neuronal overexpression experiments of Comm. Misexpressing Comm in all neurons eliminates not only Robo1 but also Robo2 and Robo3 in the CNS (Rajagopalan, Vivancos et al. 2000[a]). However, it is unclear if this sorting of Robo2 and Robo3 has any physiological relevance. Especially genetic experiments with double mutants suggest that Comm acts rather through Robo1 than through Robo2 and Robo3. Double mutants between *robo2 comm* and *robo3 comm* result in a *comm* phenotype with no axons crossing the midline, whereas a *robo1 comm* double mutant gives rise to a *robo1* mutant phenotype with abundant midline crossing (Rajagopalan, Nicolas et al. 2000[b]). Additionally, it seems that Robo2 in *robo1*^{robo2} and Robo3 in *robo1*^{robo3} are sorted as Robo1 since their expression patterns in early and in late stages resemble the wildtype *robo1* pattern (data not shown). Still, we can not exclude that there are some subtle alterations in Robo2 and Robo3 sorting through Comm which account for their inability to substitute Robo1. Finally, there are two rather likely candidates for defining the ability of midline repulsion: the intracellular conserved consensus sequences cc2 and cc3 which both Robo2 and Robo3 lack. Secondary messengers which bind to cc2 and cc3 might convey Robo1`s unique functions. In order to define which of these domains are really involved in Robo1`s unique midline repelling functions we tried to map this activity to either the extra- or intracellular domains generating chimeric receptor constructs. Unfortunately we were not able to resolve the gene-duplication of both constructs in the *robo1* locus, which is caused by the ends-in strategy of homologous recombination.

Another way to map Robo1`s repulsive function to one or several of its domains would be to generate chimeric Robo1-Robo3 receptors. Robo3 obviously has no function in midline repulsion and its domains would serve as fill-ins to keep the structure of the chimeric receptor intact. This strategy is possible since it has been shown that Robo receptors (as the Frazzled-receptor as well) are modular and domains can be functionally replaced (Bashaw and Goodman 1999). Like this, chimeras with different Robo1 and Robo3 domains can be generated. Injecting these chimeric receptor constructs into specific landing-sites would express each one of them in the same way in different flies. These flies recombined to the *robo1* mutant flies would now enable us to ask which of the domains of Robo1 when compared to the fill-in Robo3 domains can rescue the *robo1* mutant phenotype, thus enabling us to map the Robo1 repulsive functions to one or several domains.

To summarize, we can say that we confirmed Robo1`s main contribution to midline repulsion. Further we add that Robo1 is unique for this function because neither Robo2 nor Robo3 can replace Robo1 in this specific process. Only Robo2 has a minor contribution to midline repulsion and this is encoded in its genetic locus. Thus, Robo2`s contribution to midline repulsion is not specific to its protein-domains because it can be fully replaced by Robo.

Robo2 has a unique function in promoting midline crossing

In *Drosophila*, axons are repelled through Robos and Slits, but axons also have to be attracted towards the midline in order to form the neuropil. Crossing the midline is promoted through the redundant ligands NetrinA and NetrinB. They are secreted from the midline and recognized by their ubiquitous receptor Frazzled (Harris, Sabatelli et al. 1996; Kolodziej, Timpe et al. 1996). But evidence arose that Netrins are not the only attractive system at the midline. Firstly, removing both Netrins from the midline results in only about 20% of the segments in a commissureless phenotype (Harris, Sabatelli et al. 1996; Mitchell, Doyle et al. 1996). This somewhat surprising result always implied that something other than Netrins guides axons across the midline in the $NetAB^{\Delta}$ mutants. A further reason to assume an additional midline promoting system is that Netrins seem to work only on short distances (Brankatschk and Dickson 2006) leaving the question open how axons approach the midline from their sometimes very distant birth-places. But no experiments or screens revealed any other midline promoting receptors or ligands at the

midline guiding axons towards it. Also no repulsive receptors or ligands were discovered at the rim of the neuropil possibly pushing axons medially. A third possibility of how axons are attracted across the midline is anti-repulsion. Diminishing repulsion would render axons more potent to cross and this mechanism has been found in both flies and mammals. In flies, Robo receptors are not expressed uniformly but they are only present on the longitudinal tracts while the commissures are completely devoid of them (Kidd, Brose et al. 1998; Rajagopalan, Vivancos et al. 2000[a;] Simpson, Bland et al. 2000[a;] Rajagopalan, Nicolas et al. 2000[b]). This particular expression pattern makes the Robo receptors likely candidates for controlling crossing by repelling axons from the midline when expressed and allowing crossing when absent. This particular expression pattern is achieved through the post-transcriptional regulation of the Robo receptors by Comm which inhibits Robo1 to reach the cell membrane (Tear, Harris et al. 1996; Georgiou and Tear 2002; Keleman, Rajagopalan et al. 2002; Keleman, Ribeiro et al. 2005).

In vertebrates, axons get attracted towards the midline through Sonic hedgehog (Shh) and Netrins (Kennedy, Serafini et al. 1994; Serafini, Kennedy et al. 1994; Charron, Stein et al. 2003) and they are repelled by three Slits (*Slit1, Slit-2* and *Slit-3*) (Brose, Bland et al. 1999; Li, Chen et al. 1999; Holmes and Niswander 2001; Long, Sabatier et al. 2004). But the decision of whether to advance or to avoid the midline is not controlled by a Comm orthologue but rather unexpectedly regulated by a divergent member of the Robo receptor family which consists in mammals of Robo1, Robo2, Robo3/Rig1and Robo4/magic roundabout (Kidd, Brose et al. 1998; Yuan, Cox et al. 1999; Sabatier, Plump et al. 2004). It is Robo3/Rig1 which seems to contribute positively to midline crossing instead of signalling repulsion from it. Evidence comes from *robo3*$^{-/-}$ mutant mice which do not display more crossing neurons at the midline upon losing an expected repulsive component but rather had hardly any crossing axons (Sabatier, Plump et al. 2004). Additionally, the expression patterns of Robo1/Robo2 seem to be mutual exclusive to the pattern of Robo3. Whereas Robo1 and Robo2 are highly expressed in pre-crossing neurons, Robo3 is low. In postcrossing neurons the pattern reverses and Robo3 is high while Robo1 and Robo2 are low (Sabatier, Plump et al. 2004). Further, explant experiments showed that *robo3*$^{-/-}$ mutant commissural axons are repelled prematurely by a source of Slit (Sabatier, Plump et al. 2004) being consistent with a model where Robo3/Rig1 inhibits Robo1 signalling. Thus, Robo3/Rig1 would be the equivalent to the *Drosophila* Comm. But Comm has a very distinct structure than Robo receptors. Is their a possibility that the anti-repulsive mechanism is conserved in the Robo receptors? In other words, is Comm the only anti-repulsive molecule in flies?

Our experiments addressing the above questions come from triple mutant phenotype analyses. We removed the repulsive Robo receptors singularly in the *NetAB*$^{\Delta}$ double mutant background. Quantifying *NetAB*$^{\Delta}$ *robo1*, *NetAB*$^{\Delta}$ *robo2* and *NetAB*$^{\Delta}$ *robo3* revealed a surprising result: we identified a commissureless phenotype when we looked at the *NetAB*$^{\Delta}$ *robo2* triple mutant. No commissure formation at all happens in this triple mutant. Thus, we manipulated the so far known repulsive functions of the Robo2 receptor, and managed to get diminished commissure formation (Figure 5A in chapter 1). This rather implies that we removed a positive contribution to midline crossing then a negative one. Thus, it is Robo2 which guides axons across the midline in the absence of Netrins. Only now, when all factors are removed which are involved in promoting crossing, no axons can approach the midline anymore. Neither the triple mutants of *NetAB*$^{\Delta}$ *robo1* or *NetAB*$^{\Delta}$ *robo3* lead to the lack of all commissures (Figure 5A in chapter 1), implying that these receptors lack the positive function encoded in the protein-domains of Robo2. But only the knock-ins into the *robo2* locus can ask unambiguously whether Robo1 or Robo3 do have the same positive function when they are expressed like *robo2*. The hetero Swaps of the *robo2* locus in the *NetAB*$^{\Delta}$ double mutant background (*NetAB*$^{\Delta}$ *robo2*^{robo1} and *NetAB*$^{\Delta}$ *robo2*^{robo3}) are nearly as commissureless as the triple mutant of *NetAB*$^{\Delta}$ *robo2* (Figure 5B in chapter 1). This shows that the positive, midline crossing promoting function indeed is unique for the features of the protein-domains of Robo2 and not encoded in its genetic locus.

Why would a fly need a second inhibitor of Robo1`s repulsion in addition to its regulator Comm? It might be that Comm puts an axon into a state of where it can approach the midline but Comm can not deplete the growth-cone of all Robo1 receptor molecules. Thus, Robo2 ensures that there is no Robo1 receptor left to repel axons from the midline. Since this is only ensuring the fidelity of crossing we can see Robo2`s positive contribution towards midline crossing only in a sensitized background, where attraction is removed like in the *NetAB*$^{\Delta}$ double mutant background.

But what is the mechanism of Robo2`s midline promoting function. Is it indeed an anti-repulsive function similar to the one of Comm? From other species and tissues it is known that Robo1 and Robo2 can be bifunctional and convey repulsion and attraction. In muscles for example Slit seems to need Robo1 and Robo2 to attract mesodermal cells (Kramer, Kidd et al. 2001) and in tracheal development it has even been proposed, that Robo2 antagonizes Robo1 (Englund, Steneberg et al. 2002), like Robo3/Rig1 in mice does (Jen, Chan et al. 2004; Sabatier, Plump et al. 2004). Being inspired by these examples for a bi-functional Robo2, we could envision two plausible models. Both are

based on the proven repulsive functions of Robo1 and Robo2 (Kidd, Brose et al. 1998; Rajagopalan, Nicolas et al. 2000[b;] Simpson, Kidd et al. 2000[b]) and both assume that Robo2 is bi-functional. In the first model, Robo2 would be independent of Robo1 guiding axons via attraction towards and across the midline. The second model differs in the autonomy of Robo2. Now, Robo2 would depend on Robo1 in the way that it would inhibit its repulsive function thereby rendering growth-cones more likely to cross the midline. A genetically elegant way to distinguish between the two models would be to ask whether removing *robo2* in the *robo1* mutant background would enhance *robo1*`s phenotype. In the first model, removing an independent attractive function would result in hardly any commissures since the neurons are not attracted towards the midline any more. They lost the attraction through Netrins and the possible attraction through Robo2. In the second model, removing an anti-repulsive or anti-Robo1 function would not be able to enhance the *robo1* phenotype since here, Robo2`s positive function would depend on the already removed Robo1. These experiments have to be done in the $NetAB^{\Delta}$ double mutant background since only in this genetic background the positive function of Robo2 is visible. Another complication of the experimental setup is that both models are based on the repulsive function of Robo2 and therefore, a $NetAB^{\Delta}$ *robo1 robo2* quadruple mutant displays a *slit* mutant phenotype with no repulsion at all (Figure 5A in chapter 1). Thus, we were forced to look for a Robo2 receptor which lacks only the positive function but preserves its repulsive function. And the knock-in of Robo1 into the *robo2* locus is exactly this required receptor. It is repulsive since $robo2^{robo1}$ rescues midline crossing of the *robo2* phenotype and $NetAB^{\Delta}$ $robo2^{robo1}$ is commissureless. But $NetAB^{\Delta}$ $robo2^{robo1}$ also shows clearly that Robo1 can not guide more axons across the midline when expressed as *robo2* in the $NetAB^{\Delta}$ mutant background - and like this, Robo1 misses Robo2`s midline promoting function. Having a receptor in the *robo2* locus with only the repulsive function left and selectively having removed its positive function we can ask what happens to the commissures if we cross the $robo2^{robo1}$ knock-in into the $NetAB^{\Delta}$ *robo1* triple mutant. Do we get hardly any commissures as predicted in the first "attraction" model or is there no or little change in commissures as predicted in the second "anti-repulsion" model? $NetAB^{\Delta}$ *robo1* triple mutants have about 80% wildtype commissures $NetAB^{\Delta}$ *robo1* $robo2^{robo1}$ flies have ~ 61% wildtype commissures. This is significantly more commissures as in the $NetAB^{\Delta}$ *robo2* triple mutant with only 1.6% of commissures and indicates that the second model might be true and Robo2 acts indeed like the mammalian Robo3/Rig1 (Jen, Chan et al. 2004; Sabatier, Plump et al. 2004) and antagonizes Robo1`s repulsive function down.

We tried to map this positive Robo2 function to either the extra- or intracellular domains. For this attempt we crossed *robo2*$^{robo1\text{-}robo2}$ and *robo2*$^{robo2\text{-}robo1}$ chimeras into the *NetAB*$^{\Delta}$ double mutant background and asked which part of Robo2 might be able to rescue the *NetAB*$^{\Delta}$ *robo2* triple mutant phenotype where no axons cross. *NetAB*$^{\Delta}$ *robo2*$^{robo1\text{-}robo2}$ did not rescue at all with 4.5% of wildtype commissures. *NetAB*$^{\Delta}$ *robo2*$^{robo2\text{-}robo1}$ on the other hand re-established 27.2% of the wildtype commissures. Taking into consideration that the chimeras might be slightly compromised in their function and that midline crossing obviously requires a specific interplay of most probably very exact amounts of Robo1 and Robo2 could explain why the rescue is not complete (which would be at most about 80% of wildtype commissures like in the *NetAB*$^{\Delta}$ double mutant (Brankatschk and Dickson 2006). This partial rescue of the positive function of Robo2 shows (Liu, Patel et al. 2004) that at least a part of it might be encoded in the extracellular domain. This might be explained with a mechanism where Robo2 interacts with Robo1 extracellularly and it could be that it inhibits Slit-binding to Robo1`s Ig1 and Ig2 domains (Howitt, Clout et al. 2004; Liu, Patel et al. 2004).

The assumption of a bifunctional Robo2 receptor might explain another oddity. Misexpression of Robo2, using the UAS-Gal4 system on all neurons first leads to a condensation of the whole CNS, only when very high amounts of Robo2 are expressed, a commissureless phenotype arises (Simpson, Kidd et al. 2000[b]). Having only repulsive functions could not explain this result, but assuming an anti-repulsive function in a heterodimer with Robo1 (low Robo2 levels) and a repulsive function on its own (high Robo2 levels) would.

But our experiments need direct proof which might be difficult to achieve. A Co- immuno-precipitation could show direct interaction between Robo1 and Robo2 but it is quite unlikely to hit the exact time-point of this delicate interaction. Most probably this happens only during crossing which is a very short process of a few cells in the whole embryo. Additionally, Robo2 does not necessarily need to establish a strong connection to Robo1 in order to inhibit the binding of Slit and thus it might not be possible to detect it when it happens.

We show that Robo2 is bifunctional and has in addition to its well-known repulsive activity a unique function in promoting midline crossing. We speculate that the mechanism is an anti-Robo1 function, encoded in the extracellular domains and conveyed by inhibiting Slit binding to Robo1 and therefore inhibiting Robo1-signalling.

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Figure legends

Figure 1: Schematic of the expected Phentypes according to each Model – Quality (features of protein domains specify functions) or Quantity (level encode function) – for the *robo3robo2* hetero Swap. First row: genotype; Second row: expected FasII phenotype according to wildtype and the prediction for each model, quality or quantity (m: medial, i: intermediate and l: lateral); Third row: Modification for the genetic locus (*robo1, robo2* and *robo3*) and the respective receptor expressed in these loci represented by the differently coloured bars (blue: Robo1, red: Robo2 and green: Robo3). The black arrows depict the combined amount of all Robo receptors. A) In wildtype: three distinct fascicles form in each Robo-expression zone, medial, intermediate and lateral, there is no difference between the two models B) In the qualitative model the medial and lateral fascicle stay at their wildtype position and the intermediate fascicle follows its new Robo-Robo2 code and shifts (unfilled arrow) into the lateral Robo2 expressing zone, the amount of combined Robo receptors does not change – like it does not in C). Thus, the level dependent model predicts no shift of the intermediate fascicle and three wildtype lateral pathways emerge.

Figure 2: Schematic of the expected phenotypes according to each Model – Quality or Quantity – for the *robo3*^{robo1} hetero Swap. First row: genotype; Second row: expected FasII phenotype according to wildtype and the prediction for each model, quality or quantity (m: medial, i: intermediate and l: lateral); Third row: Modification for the genetic locus (*robo1, robo2* and *robo3*) and the respective receptor expressed in these loci represented by the differently coloured bars (blue: Robo1, red: Robo2 and green: Robo3). The black arrows depict the combined amount of all Robo receptors. A) In wildtype, three distinct fascicles form in each Robo-expression zone, medial, intermediate and lateral, there is no difference between the two models. B) In the qualitative model the intermediate fascicle follows its new Robo-only code and shifts (unfilled arrow) into the medial Robo expressing zone. But the total amount of all three Robo receptors stays the same and predicts in C) no shift of the intermediate fascicle and three wildtype lateral pathways since only the total amount accounts for the lateral position of a fascicle.

Figure 3: Schematic of the expected phenotypes according to each Model – Quality or Quantity – for wildtype and the *robo3* and the *robo2* mutants. First row: genotype; Second row: expected FasII phenotype (m: medial, i: intermediate and l: lateral); Third row: Modification of the genetic loci (*robo1, robo2* and *robo3*) represented by the either filled (wildtype) or empty (mutant) differently coloured bars (blue: Robo1, red: Robo2 and

green: Robo3). The black arrows depict the combined amount of all Robo receptors. A) In wildtype: three distinct fascicles form in each Robo-expression zone, medial, intermediate and lateral. B) In *robo3* mutants the intermediate fascicle shifts (unfilled arrow) into the medial one – predicted from both models: less Robo-receptor amounts in or loosing specific Robo3 function. C) In *robo2* mutants 25% of the lateral fascicle fails to do correct lateral positioning (red line). This can not be fully explained by both models but a combination of the two might do so. 75% of the fascicle positions correctly (black lateral lines) because Robo3 can substitute for Robo2 in this population. But there is a subpopulation of the lateral fascicle, not expressing Robo3 and these 25% do not have the possibility of replacing Robo2 with Robo3 and thus fail to position correctly. Like this, a Robo2 specific subpopulation of the lateral fascicle exists.

Figures

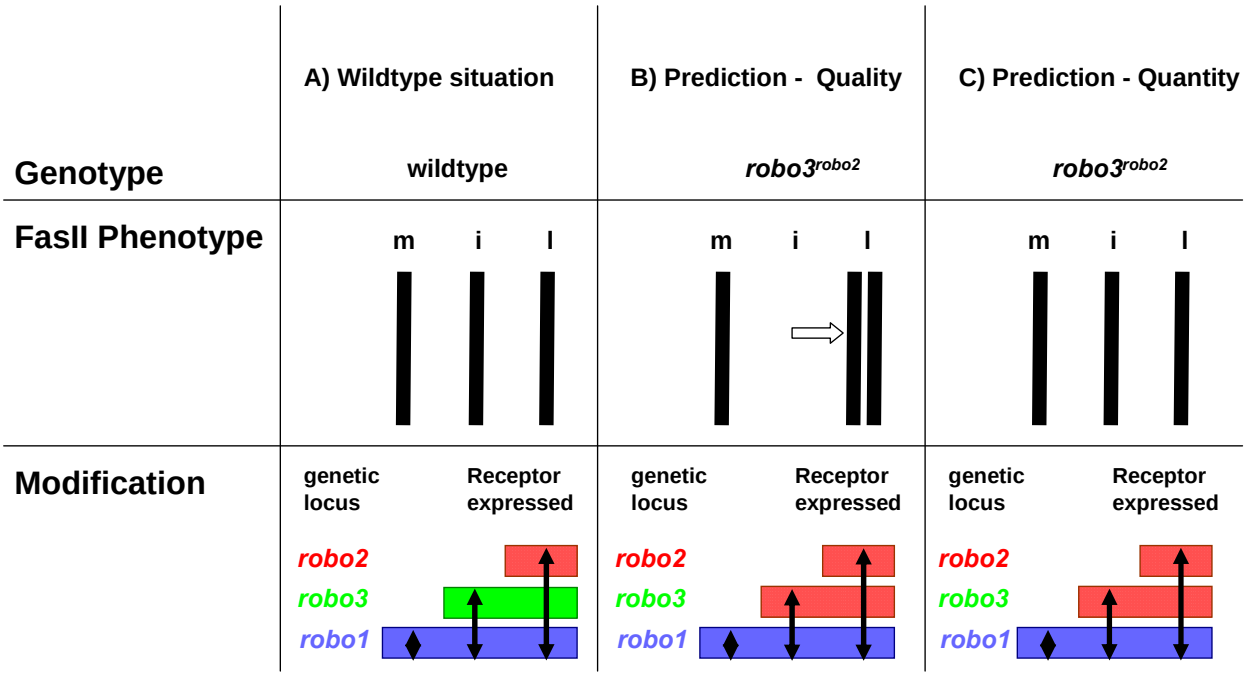

Figure 1

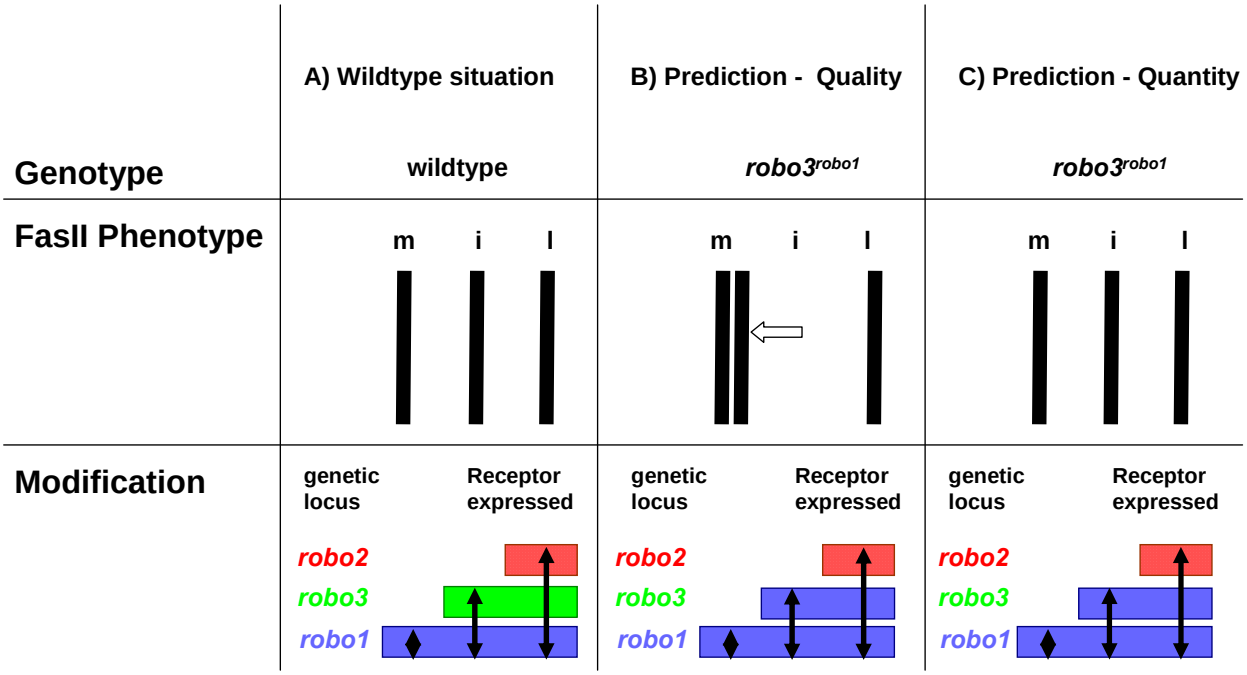

Figure 2

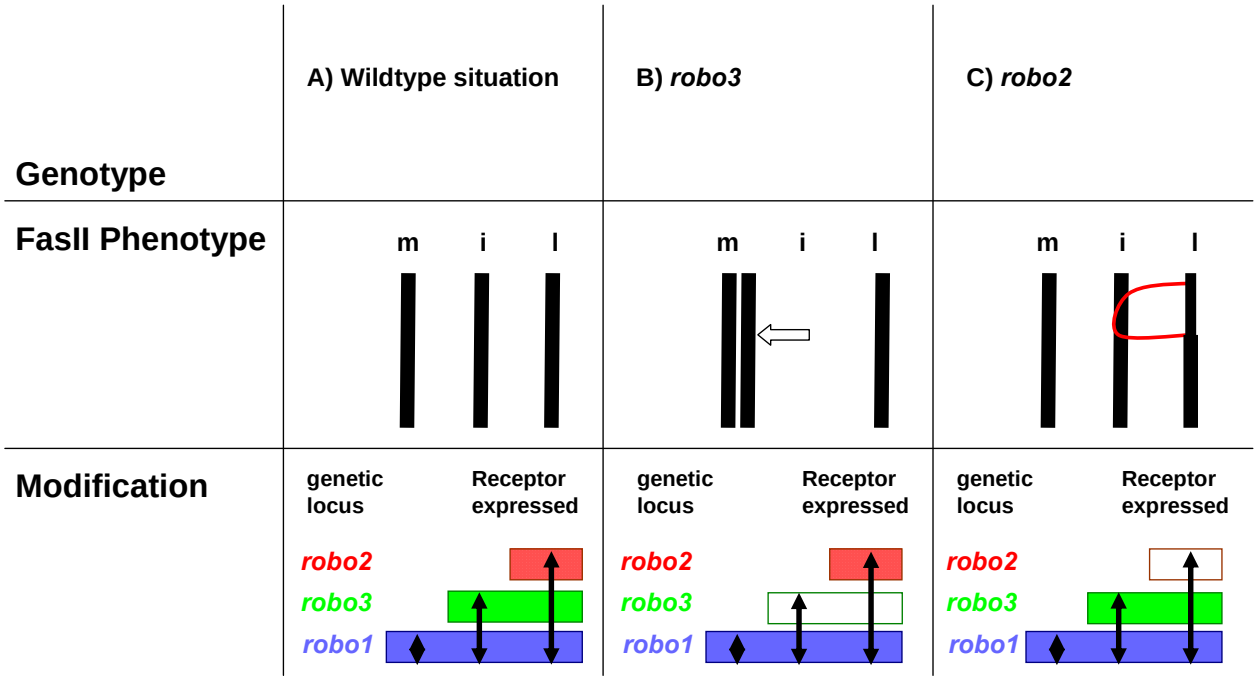

Figure 3

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