Chimeric Axon Guidance Receptors: The Cytoplasmic Domains of Slit and Netrin Receptors Specify Attraction versus Repulsion

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Summary

Frazzled (Fra) is the DCC-like Netrin receptor in Drosophila that mediates attraction; Roundabout (Robo) is a Slit receptor that mediates repulsion. Both ligands are expressed at the midline; both receptors have related structures and are often expressed by the same neurons. To determine if attraction versus repulsion is a modular function encoded in the cytoplasmic domain of these receptors, we created chimeras carrying the ectodomain of one receptor and the cytoplasmic domain of the other and tested their function in transgenic Drosophila. Fra-Robo (Fra’s ectodomain and Robo’s cytoplasmic domain) functions as a repulsive Netrin receptor; neurons expressing Fra-Robo avoid the Netrin-expressing midline and muscles. Robo-Fra (Robo’s ectodomain and Fra’s cytoplasmic domain) is an attractive Slit receptor; neurons and muscle precursors expressing Robo-Fra are attracted to the Slit-expressing midline.

Introduction

Growth cones are guided by both attractants and repellents, and these signals can be either short range or long range. Thus, there are four major guidance forces: contact-attraction, chemotraction, contact-repulsion, and chemorepulsion (Tessier-Lavigne and Goodman, 1996). With the search for guidance molecules mediating these mechanisms came the expectation that distinct families of proteins might be found that fit these categories. But with the discoveries of novel families of guidance molecules came the unexpected result that many of these molecules are bifunctional, mediating either attraction or repulsion, depending on the context. Netrins, for example, can be both attractive and repulsive (Hedgecock et al., 1990; Serafini et al., 1994; Colamarino and Tessier-Lavigne, 1995; Winberg et al., 1998a). The same now appears to be true for Semaphorins (Kolodkin et al., 1993; Luo et al., 1993; Wong et al., 1997; Bagnard et al., 1998). Most recently, compelling data have revealed multiple functions for Slits (Brose et al., 1999; Kidd et al., 1999; Li et al., 1999; Wang et al., 1999). If the ligands are intrinsically indeterminate, then the key to the sign of the response is likely to reside in either some property of the receptor or receptor complex. Alternatively, differences in the internal state of the growth cone and/or differential expression of cytoplasmic signaling components could influence the sign of growth cone response.

In the present study, we asked where attraction versus repulsion is encoded. Netrin and Slit receptors in Drosophila melanogaster provide an ideal opportunity to answer this question. In Drosophila, both Netrins (NetA and NetB) (Harris et al., 1996; Mitchell et al., 1996) and Slit (Rothberg et al., 1990; Kidd et al., 1999) are expressed by the same midline cells. At the Drosophila midline, Netrins function largely as attractants. This attractive function is mediated by Frazzled (Fra) (Kolodziej et al., 1996), a member of the Deleted in Colorectal Cancer (DCC)/UNC-40 family of Netrin receptors (Chan et al., 1996; Keino-Masu et al., 1996). Slit, on the other hand, functions as a midline repellent. This repulsive function is mediated largely by Roundabout (Robo) (Kidd et al., 1998a, 1999; Brose et al., 1999). Fra and Robo are related proteins; both are members of the immunoglobulin (Ig) superfamily. The ectodomain of Fra consists of four Ig domains followed by six fibronectin (FN) type III repeats: Fra has a cytoplasmic domain of 278 amino acids. Robo has an ectodomain of five Ig domains followed by three FN III repeats: Robo has a cytoplasmic domain of 457 amino acids. Neither cytoplasmic domain has any obvious catalytic signaling motif, but both have proline-rich regions and other short stretches of evolutionarily conserved sequences (Kolodziej et al., 1996; Kidd et al., 1998a).

Given that many growth cones near the midline simultaneously integrate attractive and repulsive signals from Fra and Robo, it seemed likely to us that the components that mediate attraction versus repulsion are ubiquitous and that the difference between attraction and repulsion is encoded in the sequence of these receptors. One model would predict that guidance receptors are modular, with ectodomains determining ligand binding and cytoplasmic domains specifying effector function. If this is correct, then swapping the cytoplasmic domains of Fra and Robo should generate chimeric receptors with the ligand binding property of one receptor and the effector function of another. If all growth cones have the necessary machinery to respond to these cytoplasmic signals, then we should be able to change growth cone behavior in vivo by transgenically driving the expression of these chimeric receptors.

To test this model, we exchanged the cytoplasmic domains of Fra and Robo and then generated transgenic fruit flies expressing these chimeric receptors on all or a subset of developing axons utilizing the GAL4-UAS two-component system (Brand and Perrimon, 1993). The model predicts that Fra-Robo should be a novel repulsive Netrin receptor and that Robo-Fra should be a novel attractive Slit receptor. In this paper, we show that both chimeric receptors behave just as predicted. Growth cones expressing Fra-Robo are repelled by Netrin-expressing midline cells, leading to a Netrin-dependent phenotype in which too few axons cross the midline. Growth cones expressing Robo-Fra are attracted to the Slit-expressing midline cells, leading to a phenotype in which too many axons cross and/or grow along the midline.

All growth cones appear capable of using these chimeric...
receptors. The chimeric receptors, when expressed at high levels, dominate the growth cone’s response and appear to overshadow the function of the normal Netrin and Slit receptors. The strength of the phenotypes is highly dose dependent. Interestingly, migrating muscle precursors expressing Robo-Fra respond to Slit in the same fashion as do Robo-Fra-expressing growth cones. Thus, although these muscle precursors are normally repelled by the midline, they contain the necessary machinery to generate the attractive response mediated by Fra’s cytoplasmic domain, even though they normally do not express Fra.

Results

 Constructs and Phenotypic Predictions

We exchanged the cytoplasmic domains of the Fra attractive guidance receptor and the Robo repulsive guidance receptor (Figures 1A–1C). The two constructs were cloned downstream of UAS activation sequences to allow targeted misexpression using various GAL4 lines (Brand and Perrimon, 1993). Based on the midline expression patterns of Netrin (Harris et al., 1996; Mitchell et al., 1996) and Slit (Rothberg et al., 1990; Kidd et al., 1999), there are clear phenotypic predictions about the consequences of pan-neural overexpression of the two chimeras. In the case of Fra-Robo (Figure 1B), we predict that growth cones expressing this chimera would be repelled by Netrin secreted from the midline glia, leading to a phenotype in which too few axons cross the midline (Figure 1E). We make the opposite prediction for Robo-Fra (Figure 1C); growth cones expressing Robo-Fra should now be attracted to the Slit protein, and too many axons should cross and/or grow along the midline (Figures 1F and 1G). As described below, our results confirm both predictions.

Pan-Neural Expression of Fra-Robo Directs Axons Away from Netrin

Flies carrying fra-robo transgenes and flies carrying elav-GAL4 were crossed together to generate animals that express Fra-Robo in all neurons during development, beginning at the time of axon outgrowth. Embryos carrying one copy of the fra-robo transgene and one copy of the elav-GAL4 driver have commissures that are significantly thinner than wild type (Figures 2A and 2B). These animals are viable and when crossed to each other generate embryos expressing higher levels of Fra-Robo. The progeny of these crosses show phenotypes ranging from wild type to completely commissureless (Figure 2C). The severity of the CNS phenotype strongly correlates with the level of Fra-Robo expression. This commissureless phenotype is consistent with the idea that the Fra-Robo receptor is interpreting Netrin as a repulsive cue and that the cytoplasmic domain of Robo can generate a repulsive response independent of the specific identity of the extracellular ligand.

In addition to guiding a subset of commissural axons

Figure 1. Summary of Chimeric Receptors and Transgenic Phenotypes

(A) The structure of the wild-type Frazzled and Robo receptors is indicated. Frazzled, shaded green, consists of an ectodomain that has four immunoglobulin (Ig) domains (broken circles), six fibronectin type III (FN III) repeats (rectangles), and a long cytoplasmic domain. It normally mediates attraction in response to Netrin at the midline. Robo, shaded red, has an ectodomain with five Ig domains, three FN III repeats, and a long cytoplasmic domain. It normally mediates repulsion in response to Slit at the midline.

(B) The Frazzled-Robo (Fra-Robo) chimera consists of the ectodomain of Fra fused to the transmembrane and cytoplasmic domains of Robo. The C-terminal six Myc tag used to monitor expression is indicated. The prediction is that this chimera should now mediate repulsion in response to Netrin.

(C) The Frazzled-Robo (Fra-Robo) chimera has the extracellular, transmembrane, and 67 amino acids of Robo’s cytoplasmic domain (red) fused to the entire cytoplasmic domain of Fra (green). The C-terminal six Myc tag is indicated. This chimera should now mediate attraction in response to Slit.

(D) The behavior of two different classes of CNS interneurons is diagrammed. The midline, which normally expresses both Netrins and Slit, is represented by a gray box. Axons of commissural interneurons (colored blue) are attracted to Netrin expressed at the midline (e.g., SP1 as shown schematically here). They cross the midline and then project ipsilaterally along it. Once they have crossed the midline, upregulation of the Robo receptor prevents them from recrossing. Axons of longitudinal interneurons (colored purple) are repelled by Slit expressed at the midline (e.g., pCC as shown schematically here). They never cross the midline but instead project ipsilaterally along it.

(E) The phenotype resulting from pan-neural expression of Fra-Robo is shown. Instead of being attracted to Netrin at the midline, axons of the commissural interneurons (blue) are repelled. This phenotype is similar to a commissureless loss of function.

(F) The weaker phenotype resulting from pan-neural expression of Robo-Fra is shown. Instead of being repelled by Slit at the midline, longitudinal axons (purple) are attracted to Slit and freely cross and recross the midline. Commisural axons (blue) now freely cross and recross the midline as well. This phenotype is similar to a robo loss of function.

(G) The stronger phenotype resulting from pan-neural expression of Robo-Fra is shown. This phenotype is driven by increased expression. All axons enter and then grow along the midline. This phenotype is similar to a slt loss of function.
across the midline, Netrin also guides certain motor axons to their correct muscle targets (Mitchell et al., 1996; Winberg et al., 1998a). NetB (one of the two Drosophila Netrins) is expressed by ventral muscles 7 and 6, where it provides an attractive cue to growth cones of the RP3 motoneuron. We examined the motor projections in embryos expressing high levels of Fra-Robo with a monoclonal antibody against Fasciclin II (Fas II; 1D4) (Van Vactor et al., 1993) to determine if there are defects in innervation of muscles 7 and 6 and/or other motor axon guidance defects consistent with Netrin acting as a repulsive cue. We observe a dramatic reduction in innervation at muscles 7 and 6 (78%, n = 155), as well as other defects in the intersegmental nerve b (ISNb) pathway (predominantly bypass and stalls) as compared to wild type, suggesting that the normal Netrin expression by these two muscles is repelling the Fra-Robo-expressing motor growth cones (Figures 2D and 2E). In contrast to fra and Netrin mutants, in which the ISN frequently crosses the segment border and fasciculates with the ISNb in the adjacent segment, we do not observe this phenotype in fra-robo gain-of-function animals.

We also see defects in the projection of the transverse nerve (TN). In wild-type embryos, a peripheral neuron, the LBD cell, projects one axon distally toward the alary muscle and another axon proximally toward the CNS. Early in stage 16, the proximally projecting LBD axon meets and fasciculates with one of the distally projecting TMN axons to form the TN (Figure 2D). This occurs near the segment border just adjacent to muscle 7 (see Winberg et al. [1998a] for details and references). The projection of the TN is not affected in Netrin or fra mutants. However, in embryos expressing high levels of Fra-Robo, the TN fails to form over muscles 7 and 6, suggesting that these axons are also now repelled by the Netrin expressed by muscles 7 and 6 (Figure 2E); both branches (the LBD and the TMN axons) stall and do not enter the region of the ventral muscles (75%, n = 148). This phenotype is very similar to what is seen when the chemorepellent Semaphorin II (Sema II) is ectopically expressed by these muscles (Winberg et al., 1998a).

It is important to distinguish between true gain-of-function phenotypes of the chimera and the potential dominant-negative effects of overexpressing the Fra extracellular domain. The dramatic difference between the Fra-Robo expression phenotypes and the fra loss-of-function phenotypes argues against the dominant-negative hypothesis. If Fra-Robo were a dominant negative for fra function, we would expect its phenotype to mimic fra loss of function. However, fra-robo phenotypes are stronger and qualitatively different than fra loss-of-function phenotypes, both in the CNS and the periphery. Furthermore, overexpression of a truncated Fra receptor (see Experimental Procedures) does not generate dominant phenotypes (data not shown). Taken together, these observations suggest that Fra-Robo is not a Fra dominant negative but rather that it senses Netrin as a repulsive cue.

The Fra-Robo Expression Phenotype Is Dependent on Netrin but Not on Frazzled

Since the fra-robo gain-of-function phenotype is considerably stronger than either the Netrin or fra loss-of-function phenotypes, we wanted to determine whether either of these two mutations would be epistatic to fra-robo. A strong prediction of the model that the Fra-Robo receptor is interpreting Netrin as a repulsive cue is that the commissureless phenotype should be dependent on Netrin but not on fra function. Therefore, we examined the effects of removing Netrin or fra function from fra-robo gain-of-function embryos.
Netrin and fra mutants display similar CNS loss-of-function phenotypes in which too few axons cross the midline and there are occasional breaks and general disorganization in the longitudinal tracts (Figures 3A and 3B) (Harris et al., 1996; Kolodziej et al., 1996; Mitchell et al., 1996). Both mutations affect the posterior commissure more strongly than the anterior (Figures 3A and 3B). In contrast, Fra-Robo expression gives a completely commissureless phenotype (Figure 3C). Removing Netrin function with a deficiency that takes out both Netrin genes (Winberg et al., 1998a) in combination with fra-robo results in a Netrin phenotype (Figure 3D), allowing significant formation of many commissures (see Experimental Procedures for details). In contrast, removing fra function has no effect on the fra-robo commissureless phenotype; rather, the two phenotypes appear to be additive: commissures do not form, but breaks are observed in the longitudinal connectives (Figure 3E). Together with the phenotypic evidence, these genetic observations further argue that the Fra-Robo chimera responds to Netrin as a repulsive cue.

Overexpression of Comm Suppresses the fra-robo Gain-of-Function Phenotype
In contrast to the endogenous Fra receptor, which is expressed uniformly on all CNS axons (Kolodziej et al., 1996), the endogenous Robo receptor exhibits a striking localization pattern. It is expressed at high levels on longitudinal axons but is greatly reduced or absent on commissural axons (Kidd et al., 1998a). This localization appears to reflect posttranslational regulation, because the same restricted expression pattern is observed when a Robo cDNA lacking untranslated regions is overexpressed in all neurons (Figure 4A) (Kidd et al., 1998a).

The presence of Robo protein in the commissures of flies carrying hypomorphic mutations in the commissureless (comm) gene indicates a role for comm in preventing Robo accumulation in the commissures. Genetic and transgenic analyses have revealed that a critical function of the Comm protein is to downregulate Robo protein levels on axons that cross the midline (Kidd et al., 1998b). It is not known what part of the Robo protein responds to Comm regulation, nor is it known what part of the Robo protein confers restriction to the longitudinal axon tracts.

To determine whether this regulatory information is present in the cytoplasmic domain (or transmembrane domain) of the Robo receptor, we examined the localization of the Fra-Robo receptor using the C-terminal Myc epitope tag. We chose a weakly expressed insert of the fra-robo transgene because embryos carrying this insert do not display any mutant phenotype (data not shown). Like the endogenous Robo receptor, the Fra-Robo receptor is restricted to the longitudinal axon tracts (Figure 4B). This suggests that the cytoplasmic domain (or transmembrane domain) of Robo can confer longitudinal localization.

The longitudinal localization of Fra-Robo suggested that Fra-Robo might be regulated by Comm. To test this hypothesis, we simultaneously expressed Comm and Fra-Robo in all neurons. We find that the fra-robo gain-of-function phenotype is significantly suppressed (Figures 4C and 4D; see Experimental Procedures). Furthermore, anti-Myc staining reveals that in embryos overexpressing both fra-robo and comm, the axonal expression of Fra-Robo is greatly reduced (data not shown). This argues that the cytoplasmic domain (or transmembrane domain) of Robo is the target for the Comm-mediated downregulation of Robo function.

Figure 3. Epistasis Analysis of the fra-robo Gain-of-Function Phenotype
All embryos pictured are stage 16 and have been stained with mAb BP102 and filleted to reveal the CNS axon scaffold. Anterior is up.
(A) A Netrin NP5 deficiency (removing both tandem Netrin genes) embryo shows a characteristic Netrin loss-of-function phenotype. The central segment is missing the posterior commissure (arrowhead), and there are breaks in the longitudinal connectives (broad arrow).
(B) A fra null mutant embryo is phenotypically similar to the Netrin deficiency embryo. Commissures are thin or absent (arrowhead), and there are breaks in the longitudinal connectives (broad arrow).
(C) The characteristic commissureless phenotype of an embryo expressing high levels of the Fra-Robo chimera is shown.
(D) A Netrin NP5 deficiency embryo that is also expressing high levels of the Fra-Robo chimera. This animal is still completely commissureless, indicating that loss of fra function does not suppress the fra-robo gain-of-function phenotype. However, the phenotypes appear to be additive in that we observe effects of the fra mutation on the integrity of the longitudinal axon tracts (broad arrow).
(E) A fra null mutant embryo that is also expressing high levels of the Fra-Robo chimera. This animal is still completely commissureless, indicating that loss of fra function does not suppress the fra-robo gain-of-function phenotype. Thus, the Netrin deficiency suppresses the fra-robo gain of function phenotype.
dominant negative. We therefore sought to express higher levels of Robo-Fra, reasoning that a dominant-negative Robo receptor should not be able to generate a phenotype that is stronger than robo loss of function, while a receptor that was sensing Slit as an attractive cue should have no such constraint and might generate a gain-of-function phenotype that approaches the stronger slit loss of function. By creating animals with different doses of the robo-fra transgene, we were able to generate a range of different expression levels and a corresponding phenotypic series (Figures 5D–5F). At intermediate levels of expression, Robo-Fra generates a CNS phenotype slightly stronger than robo null mutants (compare Figures 5B and 5E). At even higher levels, the CNS phenotype is markedly more severe than robo mutants and approaches the midline collapse phenotype characteristic of slit mutants (Figures 5C and 5F). These observations argue that Robo-Fra is responding to midline Slit protein as an attractive cue and that the cytoplasmic domain of Fra is able to access the machinery downstream of attractive guidance independent of Netrin.

Robo-Fra Does Not Act as a Dominant Negative
Although the severity of the high-level expression phenotype of robo-fra would seem to preclude the possibility that it is acting simply as a robo dominant negative, we cannot completely exclude the possibility that it is acting negatively. The difference between the robo receptor loss-of-function phenotype and the slit ligand loss-of-function phenotype suggests that there is at least one other Slit receptor in Drosophila. Indeed, there is another Robo-related protein, Robo2, that is a very good candidate to be a second Slit receptor (J. Simpson, T. Kidd, and C. S. G., unpublished). It is possible that rather than being attracted to Slit, the Robo-Fra receptor is a powerful dominant negative that blocks the function of both Robo receptors. In the case of the fra-robo chimera, the differences between the chimeric gain-of-function phenotype and the fra and netrin loss-of-function phenotypes allowed us to use epistasis analysis to confirm that the fra-robo gain of function is dependent on Netrin function and is not a Fra dominant negative. In the case of robo-fra, the similarity between the robo-fra gain-of-function phenotypes and the slit and robo loss-of-function phenotypes prohibits a similar genetic analysis. Therefore, in order to address the possibility that Robo-Fra acts as a dominant negative, we generated a Myc-tagged truncated Robo construct that has exactly the same Robo sequences as Robo-Fra but none of the Fra sequences (see Experimental Procedures). We directly compared the expression phenotypes of robo-fra with those of the truncated Robo construct roboΔC. We used the Fas II monoclonal antibody (mAb; 1D4) because it stains a subset of axons that normally do not cross the midline (Figure 6A) and therefore provides a sensitive measure of whether axons are inappropriately crossing the midline. In addition to comparing the effect of pan-neural expression (elav-GAL4) of these constructs, we also compared the effects of expressing the two constructs with the fushi tarazu neurogenic element driving GAL4(elt> GAL4), which drives expression in a subset of neurons whose axons do not normally cross the midline.
None of the four inserts of roboΔC exhibit any CNS phenotype when expressed in single copy with elav-GAL4, while in the case of robo-fra, all of the inserts give strong CNS phenotypes in single copy. In addition, anti-Myc antibody staining reveals that the robo-fra and roboΔC transgenes are expressed at comparable levels and show similar degrees of axonal expression (data not shown). At higher levels of expression, dominant-negative effects of roboΔC are revealed and a robo-like phenotype results (Figure 6B). These phenotypes are not nearly as severe as those observed for robo-fra (Figure 6C) and never exceed the robo loss-of-function phenotype. Indeed, all of the robo-fra transgenes give comparable or stronger phenotypes in single copy than the strongest roboΔC transgene when expressed in double copy (data not shown). Finally, we compared the effects of expressing the chimera and the dominant negative with ftzΔ2-GAL4. We find that the highest dose of roboΔC transgene in ftzΔ2 neurons results in only very few axons inappropriately crossing the midline (Figure 6D), while ftzΔ2 neurons expressing Robo-Fra readily cross or grow along the midline even at relatively low transgene dosages (Figure 6E and data not shown). The difference between the effects of roboΔC in the two different cases examined could reflect a difference in the susceptibility of the ftzΔ2 neurons to effects of the dominant negative. Perhaps since ftzΔ2 neurons require Robo to prevent them from extending axons across the midline, they may represent a subset of neurons that normally expresses relatively higher levels of Robo and thus may be more resistant to the dominant negative.

Mesodermal Expression of Robo-Fra Causes Muscles to Invade the Midline

In addition to functioning as a short-range repellent for axons navigating near the midline, Slit also functions as a long-range chemorepellent for migrating muscle precursors (Kidd et al., 1999). In wild-type Drosophila embryos, myoblasts initially migrate laterally away from the midline over the inside surface of the developing CNS. Later in development, some muscles extend back toward the ventral midline beneath (i.e., outside of) the developing CNS, attaching to the epidermis at a distance from the midline (Lewis and Crews, 1994). To determine whether expressing the Robo-Fra chimera on muscle precursors would affect their migration, we used the pan-mesodermal GAL4 line 24B-GAL4 to drive Robo-Fra expression. In contrast to wild-type embryos (Figure 7A), in embryos overexpressing Robo-Fra on all muscles, muscle precursors are observed extending toward and across the midline (Figures 7C and 7D). In addition to showing abnormal migration, the muscle precursors invading the midline also have unusual morphology, sending out many long thin filopodial and flattened lamellipodial extensions toward the Slit-expressing midline, suggesting that they are now attracted to...
Figure 6. Robo-Fra Generates a Gain-of-Function Phenotype that is Quantitatively and Qualitatively Different from the Robo Dominant Negative

Stage 15-16 embryos were stained with a mAb to Fas II (1D4). Anterior is up.

(A) A wild-type embryo stained with mAb 1D4. Fas II expression is observed in a subset of longitudinal axons that never cross the midline.

(B) An embryo carrying two copies of the robo\(\Delta C\) transgene and two copies of elav-GAL4. Axons of the medial-most longitudinal pathway can be seen inappropriately crossing the midline (arrowheads). This phenotype is similar to the phenotype of robo loss-of-function mutants.

(C) An embryo carrying two copies of the robo\(\Delta C\) transgene and two copies of ftzng-GAL4. In one segment, a bundle of axons of the medial-most pathway has inappropriately crossed the midline (arrowhead). This phenotype is much milder than that seen in the embryo in (B).

(D) An embryo carrying two copies of robo-fra and two copies of elav-GAL4. Axons from all three longitudinal pathways show abnormal trajectories. The severity of the phenotype shows some segmental variability. Some segments show a milder phenotype characteristic of robo mutants (arrowhead), while others show a more slit-like phenotype where several longitudinal pathways have collapsed in and are growing along the midline (broad arrow).

(E) An embryo carrying two copies of robo-fra and two copies of ftzng-GAL4. As in (D), some segments show a milder robo-like phenotype (arrowhead), while others are more compressed with multiple pathways growing along the midline (broad arrow). A comparison of the robo\(\Delta C\) phenotypes in (B) and (C) with those of robo-fra in (D) and (E) shows that robo-fra phenotypes are much more severe, affecting multiple longitudinal pathways, than those of robo\(\Delta C\).

Slit (Figures 7C and 7D). Muscles at a distance from the midline, such as muscles 7 and 6, develop quite normally. The fact that only those muscles that are navigating near midline sources of Slit show these abnormal phenotypes and that expressing a normal Robo receptor or robo\(\Delta C\) does not produce this effect argues that this phenomenon is specific to the chimera (Figure 7B and data not shown). These results support the conclusion that the Robo-Fra chimera can respond to Slit as both a long- and short-range attractant. Furthermore, these observations suggest that migrating muscle precursors use downstream signaling machinery similar to that of growth cones to respond to guidance cues in their environment.

Discussion

In this paper, we have shown that the cytoplasmic domain of a guidance receptor can determine the nature of the growth cone’s response in vivo, independent of the ectodomain and its particular ligand binding. Fra (the DCC family receptor in Drosophila), which normally mediates an attractive response to Netrin, can instead mediate a repulsive response to Netrin when given the cytoplasmic domain from Robo. In contrast, Robo, which normally mediates a repulsive response to Slit, can instead mediate an attractive response to Slit when given the cytoplasmic domain of Fra. Thus, there is nothing intrinsically attractive or repulsive about a particular guidance signal, nor is there any information in the particular ligand-binding property of a receptor’s ectodomain. Rather, and perhaps not surprisingly, the nature of the response is encoded in the cytoplasmic sequence. In short, these two guidance receptors are modular and their ectodomains and cytoplasmic domains are interchangeable.

In a companion paper (Hong et al., 1999 [this issue of Cell]), an independent in vitro study has come to a similar conclusion. Netrin receptors of the DCC family mediate attraction, while those of the UNC5 family mediate repulsion; in at least some cases, DCC proteins are required for UNC5 effects. A ligand-gated association between the cytoplasmic domains of UNC5 and DCC converts Netrin-mediated attraction to repulsion. A major conclusion of this study is that repulsion is encoded in the UNC5 cytoplasmic domain.

In Drosophila, the same midline cells normally secrete both Netrins and Slit. Growth cones can simultaneously respond to both ligands in a cell-specific fashion. Some growth cones express high levels of Fra and low levels of Robo, and they extend toward and across the midline. Other growth cones appear to express high levels of both receptors, and they can extend toward the midline, but they do not cross it (Kidd et al., 1998b). Growth cones can dramatically change their levels of Robo expression; once they cross the midline, growth cones increase their level of Robo, a change that prevents them from crossing the midline again (Kidd et al., 1998a, 1998b).
Muscle precursors can be seen inappropriately growing toward the Slit-expressing cells. These muscles have unusual morphology with many thin filopodia extending toward the midline. Anterior is left and dorsal is up.

(D) A view of the midline of the embryo shown in (B). We observe muscle precursors of unusual morphology extending toward the midline. These muscles have unusual morphology with many thin filopodia extending toward the midline. Anterior is left and dorsal is up.

Muscle precursors can be seen inappropriately growing toward the Slit-expressing cells. These muscles have unusual morphology with many thin filopodia extending toward the midline. Anterior is left and dorsal is up.

Such complex and dynamic behavior requires growth cones to be able to simultaneously respond to both attractants and repellents and to integrate these signals and respond to the relative balance of forces. Introducing a chimeric receptor into this finely tuned system leads to dramatic phenotypes. Adding a receptor that responds to Netrin as a repellent leads to a comm-like phenotype in which too few axons cross the midline. Adding a receptor that responds to Slit as an attractant leads to the opposite robo- or slit-like phenotypes, in which too many axons cross the midline or remain at the midline, respectively. These phenotypes are dose dependent, suggesting that by adding more chimeric receptor, we can tip the relative balance and thus selectively control the growth cone’s response. This striking dosage sensitivity raises the possibility of using these phenotypes as the basis for genetic suppressor screens to identify signaling components that function downstream of attractive and repulsive guidance receptors.

Another finding of this study is that the signal transduction machinery for attraction and repulsion downstream of these receptors appears to be present in all neurons, and probably in all migrating muscle precursors as well. All neurons expressing Fra-Robo or Robo-Fra appear to behave the same, regardless of their environment. If they express Fra-Robo, they stay away from the midline; if they express Robo-Fra, they extend toward the midline. No other factor appears to intrinsically commit one growth cone or another to only one kind of response. The same is true for migrating muscle precursors. Normally, many of them express Robo (and Robo2; J. Simpson, T. Kidd, and C. S. G., unpublished results) and migrate away from the Slit-expressing midline (Kidd et al., 1999). However, given the opportunity (by transgenic expression of Robo-Fra), they clearly contain the full machinery for the opposite response. In all of our transgenic experiments, the growth cone or muscle response always correlated with the level of receptor.

Here we have shown that changing the receptor composition in vivo can alter the sign of a growth cone’s response. Other recent studies have shown that changing the levels of cyclic nucleotides in vitro can lead to profound changes in the sign of a growth cone’s response. Growth cones of embryonic Xenopus laevis spinal neurons grown in vitro exhibit chemotaxis toward Netrin-1 but show chemorepulsion in the presence of a competitive analog of cAMP or an inhibitor of protein kinase A (Ming et al., 1997). In contrast, Sema III, which normally is a repellent for growth cones of Xenopus spinal neurons in vitro, instead is converted to an attractant by activation of cGMP (Song et al., 1998).

These discoveries have important clinical implications because they suggest that cyclic nucleotides may be targets for therapeutics designed to block the inhibition of nerve regeneration. This potential is further supported by a recent study showing that increasing cAMP can block the ability of both myelin and myelin-associated glycoprotein (MAG) to inhibit axon regeneration in vitro (Cai et al., 1999). But these studies on cyclic nucleotides leave open the question of whether such dramatic changes in cyclic nucleotides normally occur in growth cones and whether they are used during development to determine the attractive versus repulsive response of growth cones.

The data presented here suggest that a key factor controlling attraction versus repulsion is the receptor's cytoplasmic domain. Different cytoplasmic domains or changes in cyclic nucleotides are not mutually exclusive explanations for how growth cone attraction versus repulsion is specified. For example, differences in the signaling output of the cytoplasmic domains of specific guidance receptors could lead to different effects on...
cyclic nucleotide levels in the growth cone, which in turn could determine attraction versus repulsion. Alternatively, cyclic nucleotides could play an important modulatory function in helping to adjust the relative levels of responsiveness to different signals. The dramatic reversals of growth cone behavior observed in vitro may result from alterations in cAMP or cGMP levels beyond the normal physiological range occurring in growth cones.

The finding that the cytoplasmic sequence determines the response of a guidance receptor raises a number of interesting questions. Attraction might lead to a local change favoring actin polymerization over depolymerization, while repulsion might lead to the opposite change. But is guidance that simple? We now know the cytoplasmic sequences of five different families of repulsive guidance receptors: UNC-5s (Leung-Hagesteijn et al., 1992; Hamelin et al., 1993), Eph receptors (Cheng et al., 1995; Drescher et al., 1995), Neurotlinps (Chen et al., 1997; He and Tessier-Lavigne, 1997; Kolodkin et al., 1997), Pleins (Comeau et al., 1998; Winberg et al., 1998b), and Robos (Kidd et al., 1998a). Interestingly, they appear to share little if any sequence similarity to one another in their cytoplasmic domains. It is possible, of course, that they bind different adapter proteins that converge on the same repulsive motility machinery. But it is equally likely that not all repulsion is the same and that different classes of repulsive receptors mediate different types of responses in the growth cone. It could be that what we lump together under the term “repulsion” are actually several molecularly distinct mechanisms that negatively influence local growth cone behavior. Just what these different cytoplasmic domains do, and how many different types of repulsion exist, awaits future investigation.

Experimental Procedures

Molecular Biology

Standard procedures were used to construct the Fra-Robo, Fra.C, Robo-Fra, and Robo.C plasmids. For Fra-Robo, a 3.3 kb EcoRI/ClaI fragment containing the ectodomain of Fra, a 1.4 kb ClaI/SpeI fragment containing the transmembrane and cytoplasmic domain of Robo, and a 0.35 kb SpeI/NotI fragment containing six copies of a Myc epitope were amplified by PCR and cloned in pBluescript (pBS) to create pBS Fra-Robo-Myc. The junctions and large internal portions of the plasmid were sequenced. Fra-Robo-Myc was cloned into pUAST for transformation into flies. Fra.C-Myc was derived by internal deletion of Fra-Robo. Briefly, pBS Fra-Robo-Myc was cut to remove all but 230 nucleotides of Robo (the region encoding the transmembrane and first 67 amino acids of the cytoplasmic domain) and recircularized. The junctions were sequenced. For Robo-Fra, a Myc-tagged Robo cDNA was digested to remove all but 67 amino acids of the Robo cytoplasmic domain, creating Robo.C-Myc. A 0.9 kb Fra fragment containing the cytoplasmic domain of Fra was generated by PCR, cloned into Robo.C-Myc, and confirmed by sequencing. Robo-Fra-Myc and Robo.C-Myc were cloned into pUAST for transformation into flies.

Fly Stocks and Transgenes

The following GAL4 stocks were used: elav-GAL4, ftz-GAL4, and 24B-GAL4. At least four independent transformant lines were obtained for each construct. For the epistasis analysis of Fra-Robo, two recombinant third chromosomes were created: one that carried one copy of fra-robo and one copy of elav-GAL4 (Fr.F), and a second that carried two copies of fra-robo (FR2X). Animals that carry these chromosomes in trans are always commissureless (n > 100). This allows the Myc tag on fra-robo to be used as a molecular marker to identify the fra-robo gain-of-function animals. The following stocks and/or parental flies were created: (1) netrin+/+; Fr,E/TM2; (2) FR2X/FR2X; (3) fra<sup>asa7</sup>Cyo, elav-i-gal; Fr,E/TM2; and (4) fra<sup>asa7</sup>Cyo, elav-i-gal; FR2X/FR2X. To test the dependence of the fra-robo gain-of-function phenotype on netrin, netrin+/+; Fr,E/TM2 females were crossed to FR2X/FR2X males. Progeny were stained with Myc antibodies to identify the fra-robo gain-of-function animals and BP102 to examine the axon scaffold. The genotype with respect to NPS was either inferred from the characteristic netrin phenotype or directly examined by RNA in situ using a probe to NetB. One hundred and seven stage 15-16 embryos from the above cross were examined. Forty-one of those stained positive for Myc, indicating that they were fra-robo gain-of-function animals. Of these 41, one-fourth should be NPS animals. Thirteen of the forty-one displayed the NPS phenotype, while the other twenty-eight were commissureless, consistent with the predicted one-fourth to three-fourth ratio. For fra, fra<sup>asa7</sup>Cyo, elav-i-gal; Fr,E/TM2 females were crossed to fra<sup>asa7</sup>Cyo, elav-i-gal; FR2X/FR2X males, and progeny were stained with anti-i-gal antibodies to identify the fra-homozygous individuals and BP102 to examine the axon scaffold. Animals homozygous for fra were examined to see if any showed the commissureless phenotype associated with fra-robo gain of function. Nearly half of these animals showed the commissureless phenotype, while the other half showed the fra phenotype (34 commissureless, 39 fra-zled). Direct examination by triple staining with BP102, Myc, and i-gal antibodies confirmed these observations.

To generate animals carrying UAS commissureless and the full complement of fra-robo, we created a stock that was homozygous for UAS comm on the X and homozygous for FR2X on the third: UAS-comm/UAS-comm; FR2X/FR2X females were crossed to males who were Fr,E/TM3actTG-i-gal. Progeny were stained with anti-SexLethal (to stain females), anti-i-gal (to stain TM3 flies), and BP102 to examine the axon scaffold. Progeny that stained only with BP102 were males that were UAS-comm; Fr,E/FR2X. All of these animals showed a similar suppression of the fra-robo gain-of-function phenotype with some significant commissure formation in greater than 60% of segments (n = 29 animals).

Immunohistochemistry

Embryo-staining procedures were as previously described (Kidd et al., 1998a).

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