

Robo is Abl to block N-Cadherin function

Mark M. Emerson and David Van Vactor

The molecular pathways involved in wiring the brain have just begun to be elucidated. Work in this issue of *Nature Cell Biology* has uncovered inhibitory interactions between two such pathways: Roundabout (Robo) and N-Cadherin. This discovery provides a potential mechanistic understanding of how pathways are used in a coordinated manner to guide axons.

Biologists have long been impressed by the ability of the nervous system to wire itself into a functional unit. At the single-cell level, a neuron must generate an axonal process and extend that process over significant distances. Along the way, the axon will often make several precise changes in direction and encounter different physical and biochemical environments. The path that an axon chooses to take is presumably orchestrated by a coordinated presentation of extracellular cues by intermediate and final targets. These cues are interpreted by axonally localized receptors, adhesion molecules and their downstream effectors¹. The integration of these cues occurs in the growth cone (the motile end of the axon) and usually results in one final directional output. With some of the key guidance factors and receptors now known, research is just beginning to explore how these signal transduction and adhesion systems function.

Simple models of axon guidance propose that axons change direction because they are challenged with a new extracellular cue or cues. Given the complexity of an axon's journey, it would seem that the more cues an axon can draw on, the better. New data, however, suggest that at certain times, the ability to ignore cues is vital to accurate guidance of the growth cone. At the molecular level, such cunning ignorance can be achieved by allowing one guidance pathway to inhibit the signal transduction of another. Work from Rhee *et al.*² in this issue of *Nature Cell Biology* describes convincing evidence for a biochemical inhibition of the cell adhesion molecule N-Cadherin by the guidance receptor Robo. These new findings, together with earlier work on Robo, make for tempting speculation on how cross-pathway inhibitions observed *in vitro* could regulate axon guidance *in vivo*³. We will outline potential uses of cross-pathway inhibition in axon guidance, review the known functions of Robo and hypothesize how the discovered cross-pathway inhibition mediated by Robo adds to our mechanistic understanding of axon guidance.

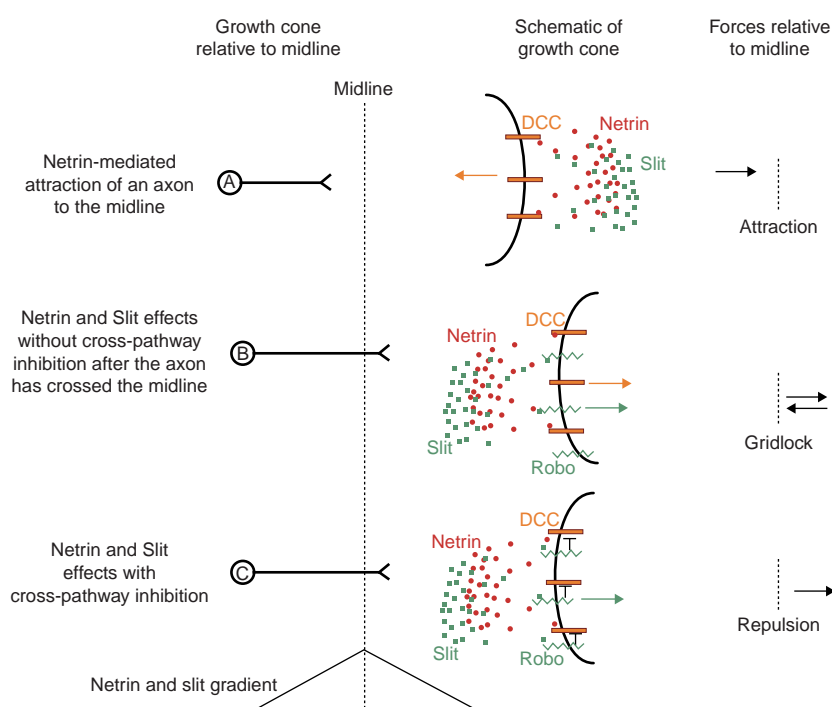


Figure 1 Cross-pathway inhibition prevents gridlock at the midline. The left column shows the growth cone relative to the midline, the middle column shows the state of Robo and DCC signalling within the growth cone, and the right column shows the supposed forces generated by these signals within the growth cone. Netrin and Slit are both ligands that are secreted from the midline. The axon of cell A is attracted to the midline through Netrin's stimulation of DCC (top). The axon of cell B has reached the midline and transduces both the Slit and Netrin signals because it now expresses Robo on its surface (middle). This cell has no cross-pathway inhibition and so suffers gridlock as the forces generated in the growth cone antagonize each other. The axon of cell C can resolve this conflict of forces by a Slit-dependent inhibition of the Netrin–DCC pathway (bottom). Only the Robo–Slit signal is propagated within the growth cone and a repellent response is generated.

The most obvious use for cross-pathway inhibition is the prevention of signal 'gridlock'. Gridlock refers to what would occur if an axon were receiving and transducing opposing signals simultaneously. These signals would work against each other,

paralysing the forward progress of the axon. However, if one signal inhibits the signal transduction of the other, this paralysis is prevented and the growth cone only uses the dominant cue to guide it. There are at least three applications for cross-pathway

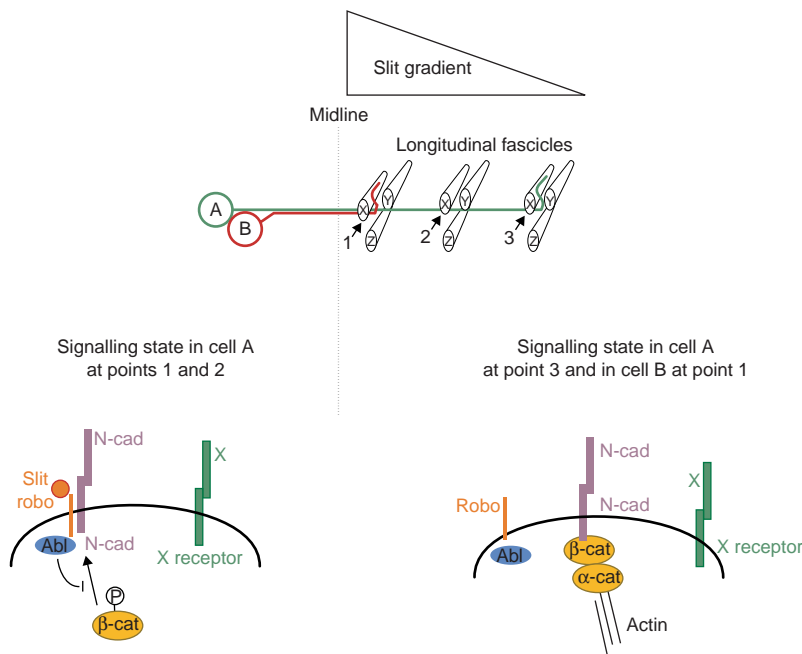


Figure 2 One possible *in vivo* role for the Robo-mediated inhibition of N-cadherin. A model for how three guidance cues—Slit, N-cadherin, and other adhesion molecules act in concert to target longitudinal axons. Axons cross the midline and do two things: first, they continue to travel various distances away from the midline; second, they choose one out of many longitudinal fascicles to join. Axon A receives a high level of Robo signalling and so moves down the Slit gradient to position 3 before choosing one of the fascicles in the area. Here, it chooses the X fascicle through its X-receptor. Axon B has a low level of Robo signalling and so can tolerate the high concentration of Slit near the midline. In common with axon B, it also chooses the X fascicle, but from this more medial cohort of fascicles. Combining the work from Rhee and colleagues with earlier work on longitudinal axon positioning suggests the signalling states shown above. At point 1, N-cadherin-mediated adhesion is disabled in axon A as a result of Slit-mediated activation of Robo. This is mediated by the formation of a complex between Robo, Abl and N-cadherin and phosphorylation of β -catenin by Abl, which blocks its interaction with N-cadherin. Although axon A can adhere to the X fascicle through the X-receptor, it needs N-cadherin to actually exert a physical force within the growth cone. Thus, signalling from Robo prevents gridlock through inactivation of N-cadherin and continues to repel the axon further from the midline. However, axon B at point 1 is not very responsive to Slit, as it has only a small amount of Robo signalling. Thus, inhibition of β -catenin is relieved. A functional N-cadherin allows for axon B to use the X cue and turn into this fascicle. Axon A must wait until point 3 for its Robo signalling to be low enough to achieve the same goal.

inhibition: to suppress a pathway that has outlived its usefulness (shutdown), to prevent an axon from straying between cues (handoff) and to suppress a pathway until the axon accomplishes other goals first (holdback). We will first give a brief description of the Robo pathway and then illustrate where these mechanisms might function.

In vivo, Robo receptors have an evolutionarily conserved function in the midline, one of the most extensively studied axon guidance choicepoints⁴. The majority of CNS axons cross the midline at some point in their journey. Netrin is a midline-secreted factor that initially attracts the axon to

the midline. After reaching the midline, axons continue their progression on the opposite side of the embryo, repelled from the midline and never venturing back across it. This repulsion is achieved by Robo receptors responding to their ligand, midline-secreted Slit. In *Drosophila melanogaster*, recent work has shown that the Slit–Robo system also has another function^{5,6,7}. Many axons continue their journey after crossing the midline by making a sharp turn and joining one of many longitudinal axon pathways that are already tracking parallel to the midline. The discovery of homophilic cell adhesion molecules such as connectin and Fasciclin II (Fas II),

which label specific subsets of these pathways), suggested that they, and presumably other molecules, might function as the cue used by axons to make their turn. Those axons with FasII on their surface join or bundle with the other axons that express FasII. One problem with this simple model is the fact that there are three major Fas II-positive bundles located at medial, intermediate and lateral positions, relative to the midline. If FasII is the only cue axons use, they should all join the medial bundle, which they encounter first after crossing the midline. Recent work has shown that axons joining these bundles express a unique combination of the three *Drosophila* Robo receptors and suggests a 'Robo code' for lateral positioning. Axons with fewer Robo-family receptors join the medial pathway because they can tolerate high concentrations of Slit. Those axons with a high degree of Robo signalling are pushed furthest from the midline and join the lateral pathway. Axons with an intermediate amount of Robo signalling join the intermediate fascicle where Slit levels are moderate. In this model, the long-range Slit–Robo system roughly targets an axon to its longitudinal neighbourhood and then short-range (that is, membrane-bound) cues target the axon to a specific fascicle^{5,6,7}.

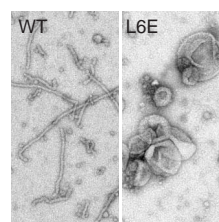
How could cross-pathway inhibition mediated by Robo help us understand its role *in vivo*? The transition from initial attraction to repulsion from the midline is a prime example of why axons need anti-gridlock mechanisms. Previous work has shown that Netrin-guided axons arriving at the midline begin to respond to the repulsive effects of Slit and are repelled into the other side of the midline⁴. But why is the axon not paralysed by the opposing forces of Slit and Netrin? The answer seems to be that Robo can directly antagonize the Netrin receptor, deleted in colorectal cancer (DCC), through receptor–receptor interactions³ (Fig. 1). This is an example of signal 'shutdown', as Robo is inhibiting a pathway that has lost its importance to the cell. It is also an example of 'handoff', because the attraction to netrin becomes a repulsion for Slit, with no gap in between. Without such a mechanism, the embryo would have to precisely coordinate three things: the down-regulation of the Netrin pathway, the upregulation of the Slit pathway and the simultaneous arrival of all axons at the midline. Cross-pathway inhibition allows axons to function independently from each other and keeps them with a target in their sights.

After axons cross the midline, they use the positional information encoded by Slit and interpreted by their Robo code to find their place in the longitudinal tracts^{5,6,7}. Given the work with Fas II, a 'holdback' form of cross-pathway inhibition would be an appealing explanation of how local cues

Bending the membrane

The budding of vesicles from membranes requires marked changes in the curvature of the membrane. How these changes are achieved and contribute to vesicle budding is an area of intense interest. One model invokes the enzymatic modification of membrane lipids, which alters membrane curvature by converting inverted-cone-shaped lipids to cone-shaped lipids. Others have proposed that proteins able to induce membrane tubulation by increasing curvature do so, at least in part, by virtue of forming oligomers on the membrane. Recent work from McMahon and colleagues now presents an attractive model for how epsin may promote the formation of clathrin-coated vesicles by directly bending the plasma membrane (*Nature* 419, 361–366 (2002)).

Epsin 1 has been implicated in clathrin-mediated endocytosis. It interacts both with phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P₂) through its ENTH domain, as well as several components of the clathrin coat, and has been suggested to recruit and promote polymerization of clathrin. When added to liposomes containing PtdIns(4,5)P₂, epsin 1 can drive the formation of tubules. To better understand how this is achieved, the authors solved the structure of the epsin 1 ENTH domain in the presence of the PtdIns(4,5)P₂ head-group, inositol-1,4,5-trisphosphate (Ins(1,4,5)P₃). This structure revealed a helix at the amino terminus of the ENTH domain (termed helix 0) that participates in the binding of Ins(1,4,5)P₃ but remained unstructured in a previously determined structure of the ENTH domain alone. Mutation of



helix 0 residues severely affects the ability of the ENTH domain to tubulate liposomes: a larger hydrophobic surface enhances the ability of epsin to tubulate liposomes, whereas a smaller hydrophobic surface reduces this ability (see figure). In an assay employing lipid monolayers containing PtdIns(4,5)P₂, epsin is sufficient to cause clathrin-coated membrane invagination, and this activity is also critically dependent on the hydrophobic surface of helix 0.

How might helix 0 contribute to the induction of membrane curvature? As an amphipathic helix, helix 0 has the potential to interact with the membrane through its hydrophobic surface. The authors therefore suggest that helix 0 is inserted into the outer leaflet of the lipid bilayer, where it pushes the head-groups apart, thereby bending the membrane. They further speculate that the recruitment of clathrin and subsequent formation of a clathrin cage may stabilize this initial increase in membrane curvature. Adding to the existing ideas, this exciting new model will no doubt spur further research into the understanding of the forces underlying vesicle formation.

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are prevented from prematurely engaging axons. Robo could inhibit the sensing of local cues until the axon is far enough away from Slit to reduce its Robo signalling. This would relieve Robo-mediated inhibition of short-range cues and allow the appropriate longitudinal tract to be joined. Although no biochemical link has been found between Robo and Fas II, the work from Rhee *et al.*² uncovers a biochemical inhibition between Robo and another well-known short-range cue, N-cadherin.

N-cadherin is a homophilic cell adhesion molecule that regulates oriented axon outgrowth both *in vivo* and *in vitro*^{8,9}. The work of Rhee *et al.* begins with the observation that activation of the Robo signalling cascade caused a specific decrease in N-cadherin-mediated adhesion². The authors begin to fill in the molecular gaps by first examining the role of N-cadherin-associated proteins in the Robo response. In the adhesive state, N-cadherin is bound to β -catenin, which is itself bound to α -catenin, which in turn binds F-actin¹⁰. This bridge from N-cadherin to the actin cytoskeleton seems to be necessary for N-cadherin-mediated adhesion, as loss of the interaction between N-cadherin and β -catenin causes loss of adhesion^{11,12}. Rhee *et al.* found that

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after activation of Robo, N-cadherin no longer immunoprecipitated β -catenin². This loss of interaction is coincident with an observed increase in tyrosine-phosphorylated β -catenin. The authors go on to show that the tyrosine kinase Abelson (Abl) is constitutively associated with Robo and is the most probable candidate for phosphorylating β -catenin. Activation of Robo results in the formation of a complex consisting of Robo, Abl and N-cadherin, which

may facilitate the tyrosine phosphorylation of β -catenin². Although this is an *in vitro* study, there is existing evidence for some of these interactions *in vivo*. For example, in *Drosophila*, Abl interacts genetically with β -catenin and DE-Cadherin in various morphogenetic contexts^{12,13}. Work in *Drosophila* has also suggested a genetic interaction for Abl and Robo, but one in which Abl inhibits the Robo pathway¹⁴. The work from Rhee *et al.* predicts that there will also be a positive role for Abl in Robo signalling, consistent with more recent studies¹⁵.

What part might cross-pathway inhibition of N-cadherin by Robo play in axon guidance? One intriguing possibility is that it cooperates with the Robo–Slit pathway in the positioning of longitudinal pathways. The original ‘Robo-code’ model suggested that axons are first targeted by the Robo–Slit pathway and then local adhesion cues, such as Fas II, guide the axon to a final position^{5,6,7}. This model, however, does not address the issue of gridlock. How is it that axons targeted to the lateral tract by their Robo signalling are not paralysed by the positive local adhesion cues in the medial and intermediate pathways that they must traverse? One possibility is that activation of Robo causes cross-pathway inhibition of all

the local adhesion cues by inactivating each one individually. A much more attractive hypothesis is that Robo inhibits a common factor that is necessary for the axon to migrate in a longitudinal pathway, while the local adhesion cues determine the specificity of pathway selection. *Drosophila* N-Cadherin (DN-Cadherin) is an excellent candidate for just such a common factor. It is found throughout the embryonic nervous system and when mutated, it causes defects in longitudinal axon extension⁹. Cues such as Fas II could be responsible for the initial targeting and maintenance of axon fasciculation, whereas DN-cadherin could be the link to the cytoskeleton that would drive extension of the axon. Thus, the inhibition of DN-Cadherin by Robo would make the axon unable to follow any

longitudinal pathway cues until the proper level of Robo–Slit signalling for that axon was achieved (Fig. 2). This fits well with the reported phenotype of Fas II mutants, whose axons still extend longitudinally, but fasciculate inappropriately with other axons¹⁶. Whether the mechanism uncovered by Rhee *et al.*² is involved in a holdback scenario such as this, or some other form of cross-pathway inhibition, remains to be seen. It suggests, however, that the interactions between pathways may be as important as the pathways themselves in creating a properly wired brain. □

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A glimpse of coated vesicle creation? Well almost!

Francesca Santini and James H. Keen

The budding of a cargo-laden clathrin-coated pit (CP) from the plasma membrane (PM) during receptor-mediated endocytosis is a paradigm of vesicular transport. A recent study published in *Nature Cell Biology* helps us visualize the creation of the clathrin-coated vesicle, and the involvement of dynamin and actin as potential ‘midwives’ in the process. Still, it remains a matter of faith as to exactly when life begins for the coated vesicle.

During endocytosis, a variety of cargo molecules and cell-surface receptors (such as the low-density lipoprotein receptor and the transferrin receptor) are concentrated into clathrin-coated regions of the PM. These regions invaginate to form pits and then eventually detach from the surface. After rapidly losing their coats, endocytic vesicles begin a complicated intracellular journey during which their contents are either sorted to appropriate destinations on the endocytic pathway or recycled back to the PM. Initially, the budding of coated vesicles seemed to be a comparatively simple process, involving the formation of a complex between clathrin, AP-2 and one of a number of cell surface receptors. AP-2 seemed to function as an adaptor to bring the receptors into contact with the internalization machinery. However, new methodologies — such as yeast two-hybrid analyses, glutathione S-transferase pull-down experiments, viral panning and database homology searches — have identified more than 30 proteins that bind either directly or indirectly to this triad complex¹.

Of particular relevance here, dynamin (which has been implicated either as a ‘pinchase’ or a regulator of CP detachment²) and actin are thought to be involved in this process (reviewed in ref. 3). So, the imperative is to order all these factors in the time (1 min) and space (0.2 μ m) that defines the life of a CP within an intact cell.

One approach that helped to decipher the dynamics of functional CPs came several years ago, when it was shown that green fluorescent protein–clathrin (GFP–clathrin) could function as a reporter for the behaviour of CPs in live cells. A ‘blinking’ pattern of GFP–clathrin dots was observed, which was consistent with the formation of CPs at discrete sites, their persistence for periods of a minute or so, then rapid disappearance and subsequent reappearance⁴. In a recent issue of *Nature Cell Biology*, Merrifield *et al.* have now taken this approach to study the behaviour of CPs within living cells to a new level by combining conventional epifluorescence microscopy with total internal reflection fluorescence (TIRF) microscopy⁵. TIRF is a powerful tool that reveals the

behaviour of fluorophores within a short distance from the substrate surface and the authors could reliably correlate the decreasing signal with movement of up to 70–100 nm from the substrate surface. The TIRF approach is also insensitive to more distant signals; thus, it serves as an exquisite sensor for events at the PM.

Merrifield *et al.* expressed a red fluorescent conjugate of clathrin (clathrin–DsRed) and focused on a discrete population of CPs. By capturing sequential epifluorescence and TIRF images, the total clathrin signal and the clathrin adjacent to the substrate, respectively, were resolved. Remarkably, while the epifluorescence signal of the clathrin spot remained essentially unchanged over 60–90 s at the experimental temperature (27–28 °C), the TIRF signal dropped almost to background over a period of 10–30 s. The authors propose that the start of this decline in the TIRF signal represents the initiation of the ‘departure moment’, and infer that this indicates the detachment of the CP from the PM and its escape into the cytoplasm. Through ele-