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Plexin Signaling via Off-Track and Rho Family GTPases

Two papers in this issue of *Neuron* examine new aspects of Semaphorin signaling via Plexin receptors. Winberg et al. present evidence that the transmembrane protein Off-track (OTK) interacts biochemically and genetically with Plexin A and is important for Sema 1a repulsive signaling. Hu et al. examine the coupling of Plexin B to Rac and RhoA and propose that Plexin B signaling involves inhibition of Rac function by direct sequestration and simultaneous activation of RhoA.

During the wiring of the embryonic nervous system, axonal growth cones navigate through an environment of both long- and short-range attractive and repulsive cues. Several classes of axon guidance ligands and receptors have been identified in recent years, but exactly how these cues are translated into the local alterations in the actin cytoskeleton required for changes in growth cone motility is poorly understood. Two papers in this issue of *Neuron* (Winberg et al., 2001; Hu et al., 2001) provide insight into signaling mechanisms downstream of Semaphorins.

Off-Track as a Mediator of Plexin Signals

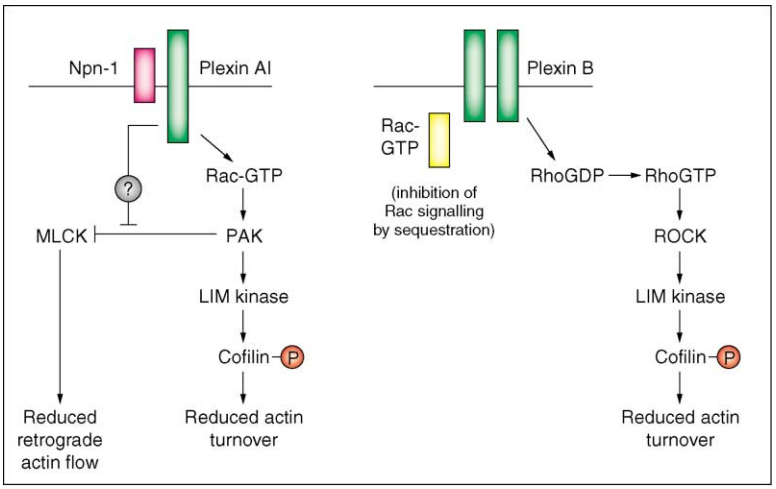
Drosophila has two Semaphorin receptors, Plexin A (PlexA) and Plexin B (PlexB) (reviewed by Tamagnone and Comoglio, 2000). It had previously been reported that Plexins were tyrosine phosphorylated in cell extracts, although they themselves have no kinase activity. Additionally, the Plexins copurified with a protein of 160 kDa, suggesting that the associated protein might be a tyrosine kinase. Winberg et al. (2001) utilized a candidate gene approach to attempt to identity this Plexin partner. They report that a putative receptor tyrosine kinase called Off-track or OTK (previously called Dtrk) interacts both in vitro and in vivo with PlexA, a receptor for the transmembrane Semaphorin Sema 1a, and appears to be involved in Plexin-mediated signaling. They demonstrate that OTK and PlexA expressed in heterologous cells can associate biochemically, suggesting that OTK may be part of the Plexin receptor complex. Among other phenotypes, *otk* mutants show defects in the guidance of the ISNb motor axon as it defasiculates and branches at specific choice points along its trajectory. These guidance defects are similar to those seen in *PlexA* and *Sema1a* mutants (Winberg et al., 1998), and transheterozygous interactions suggest that these three genes may act in a common pathway: flies heterozygous for *otk*, *PlexA*, or *Sema1a* appear normal; flies doubly heterozygous for *otk* and *PlexA* or for *otk* and *Sema1a* have ISNb defects. Surprisingly, after basing their candidate gene approach on the search for a receptor tyrosine kinase, it appears that OTK may actually be kinase dead, since the catalytic domain of the receptor is altered at a few key residues implicated in autophosphorylation. However, there is precedent—the same is true of Derailed (Drl), a kinase dead RTK critical for axon guidance in *Drosophila*. It remains to be seen if OTK recruits another protein, one with kinase activity, that phosphorylates PlexA.

While the evidence that OTK participates in Sema1a and PlexA signaling is strong, it is important to recognize that additional experiments will be necessary to firmly place OTK in the PlexA signaling pathway. Two issues deserve particular attention. First, it will be important to demonstrate that endogenous PlexA and OTK interact in neurons. Second, it is useful to remember that transheterozygote interactions do not necessarily imply that two gene products participate in a direct signaling pathway. Relevant examples in this case are experiments from the Goodman lab (Winberg et al., 1998) and the Kolodkin lab (Yu et al., 2000), which demonstrate transheterozygous interactions between PlexA and FasII and between Sema 1a and FasII, although FasII is not thought to be a direct target of Sema 1a/PlexA signaling. Independent of the details of the relationship between PlexA and OTK, the study clearly establishes that OTK has an important role in axon guidance, and further investigations of this molecule will undoubtedly reveal new insights into the molecular mechanisms of axon guidance.

Plexin Signaling via Rho GTPases

In the second paper (Hu et al., 2001), the authors examine the interactions between PlexB, the other *Drosophila* Semaphorin receptor, and members of the Rho family of small GTPases. The Rho GTPases have been extensively studied as modulators of the actin cytoskeleton. They function as molecular switches, cycling between an active GTP-bound form and an inactive GDP-bound form. They are activated and inactivated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs), respectively. Although there are at least 14 human Rho GTPases, only three, RhoA, Rac1, and Cdc42, have been extensively studied. In fibroblasts, expression of RhoA leads to the formation of stress fibers, Rac1 leads to lamellipodia formation, and Cdc42 leads to the formation of filopodia. Based on the effects of expressing combinations of constitutively active and dominant-negative (DN) forms of these GTPases, Hall and colleagues initially proposed that Cdc42, Rac1, and Rho act in a linear signaling cascade (Nobes and Hall, 1995). It is not clear, however, if such a linear signaling pathway functions in neurons, since several studies have reported that Rac1 and Cdc42 promote axon growth, while RhoA appears to inhibit growth (reviewed in Luo, 2000).

The contrasting effects of Rac and Rho activation on axonal growth led to the proposition that activation of Cdc42 and Rac1 may be associated with responses to attractive cues, while activation of RhoA may be associated with responses to inhibitory guidance cues that cause axon retraction and growth-cone collapse. This simple idea, however, was quickly placed in doubt as Jin and Strittmater and others reported that expression of a dominant-negative form of Rac1 inhibited growth



Model for Plexin Receptor Activation

Two models of how activation of Plexin receptors could lead to reduced actin turnover and growth cone collapse. In the first model, based mainly on studies of Sema3A, which acts via a Neuropilin1-PlexinA1 receptor complex, the signal is transduced by Rac-GTP. In the second model, based on studies using the Plexin B receptors, Rac-GTP is sequestered by the receptor, and the signal is transduced by Rho-GTP. Activated Rac, when expressed by itself, promotes axonal growth, while activated Rho inhibits growth. For the first model to be correct, additional mechanisms must be involved that suppress the growth-promoting effects of Rac-GTP, which involve regulation of Myosin light chain kinase (MLCK).

cone collapse in response to a soluble Sema3A, suggesting that activation of Rac1 may be involved in transmitting the Semaphorin signal (Jin and Strittmatter, 1997; Vastrik et al., 1999; Kuhn et al., 1999). Then Vikis et al. and Rohm et al. reported that the Semaphorin receptor PlexinB1 can interact directly with Rac1 but only in its GTP-bound state (Vikis et al., 2000; Rohm et al., 2000). Similar observations were made by Driessens et al. (2001), which leads to the study by Hu et al. in this issue.

Building on these earlier in vitro studies, Hu et al. first show that PlexB interacts with Rac1 and Rac2 via a seven amino acid stretch near the C terminus. They also find that PlexB can interact directly with RhoA via a distinct domain, but, unlike Rac, RhoA can interact with PlexB in either the GTP- or GDP-bound state. To determine whether this association between PlexB and Rac is functionally important, the authors tested for dosage-sensitive genetic interactions in vivo. Overexpression of PlexB leads to a number of axon guidance defects in axons innervating the muscle fields, including lack of the RP3 motor axon branch and a stall of ISNb. When levels of Rac protein are reduced by 50%, the penetrance of the PlexB gain of function (GOF) effect is increased. Correspondingly, increasing levels of Rac protein with a *UAS-Rac* transgene decreases the penetrance of the "RP3 missing" and "stall" phenotypes. Additionally, PlexB overexpression enhances the axon defects found in transgenic flies overexpressing a dominant-negative Rac, while reducing PlexB suppresses the defects. These genetic interactions are dependent on a direct association between PlexB and Rac; a mutant PlexB transgene lacking the Rac-GTP binding domain fails to enhance the DN Rac phenotype or demonstrate a gain-of-function phenotype. Taken together, these results convincingly demonstrate that PlexB antagonizes Rac output in vivo. What about the interaction between PlexB and RhoA? The authors show that mutations in *RhoA* suppress the PlexB GOF phenotype, suggesting that the effects of PlexinB activation require RhoA function, although whether PlexB activation leads to RhoA activation has not yet been demonstrated.

Since PlexB interacts with Rac only when Rac is bound to GTP, the authors propose that PlexB may sequester activated Rac away from its downstream effector PAK. In support of this possibility, the authors show that PlexB successfully competes with PAK for activated Rac in a pull-down assay. Additionally, overexpression of PAK in the PlexB GOF background suppresses the GOF phenotype, suggesting that PAK is also negatively regulated by PlexB activation. Thus, the authors propose an attractive model in which PlexB mediates chemorepulsion by sequestering Rac-GTP away from PAK and activating RhoA. The evidence for this model is quite compelling, and one imagines that some remaining important experiments are being carried out. For instance, the evidence for biochemical interactions between PlexB and Rho GTPases is based on in vitro binding assays, and it will be important to demonstrate that PlexB interacts with Rac and Rho in neurons. It will also be important to determine whether activation of PlexB indeed suppresses biochemical signaling by Rac to its downstream effectors. It is noteworthy that the mechanism proposed by Hu et al. is conceptually similar to a mechanism proposed by Michael Greenberg's group with regard to Ephrin signaling. They recently identified an exchange factor called Ephexin that interacts with the Ephrin receptor EphA4 (Shamah et al., 2001). Activation of EphA4 appears to inhibit Rac and Cdc42 and activates RhoA. Thus, opposing regulation of Rac and Rho proteins may be a common mechanism for regulating cytoskeletal changes in response to inhibitory guidance cues.

It is not yet clear whether the model proposed for PlexB signaling will apply universally to all Plexins. As noted above, earlier studies in mammalian cell culture systems suggested that Rac function is necessary for collapse in response to Sema3A. Recent work from Aizawa et al. demonstrating that Sema3A treatment leads to LIM kinase activation, which in turn can lead to phosphorylation of Cofilin, an actin binding protein, provides further insight into these signaling cascades (Aizawa et al., 2001). In fibroblasts, Rac1 can activate LIM kinase, which suggests the possibility that one of the mechanisms by which Sema3A regulates the actin cytoskeleton may involve activation of Rac1 and LIM kinase in sequence followed by Cofilin phosphorylation. Since Sema3A acts via activation of Plexin A1, this pathway

may be important for transducing signals downstream of Plexin A signaling, while the Rac sequestering mechanism may be important in transducing Plexin B signals. Hu et al. note that, unlike PlexB, PlexA does not interact directly with Rac1, which may suggest differences in signaling via these receptors. However, based on unpublished genetic evidence, the authors suggest that at least in flies PlexA and PlexB are likely to signal in a similar manner (see Figure).

Finally, it is worth noting that, although the study by Hu et al. focuses on direct interaction between PlexB and Rho family GTPases, evidence continues to accumulate that other molecules that regulate the activity of these GTPases also play important roles in specifying neuronal morphology. The most recent observations along these lines are decribed in an upcoming paper in *Cell* from the laboratory of Liqun Luo, demonstrating a role for p190 RhoGAP in axon branch stability in *Drosophila* (Billuart et al., 2001). Likewise, mice lacking p190 RhoGAP have axon guidance and fasciculation defects (Brouns et al., 2001), demonstrating the importance of RhoGAP in mammalian axon guidance.

The evidence linking Rho GTPases to axon guidance events is now quite compelling, but a number of challenges lie ahead. First, it remains to be determined how the various components that regulate Rho GTPase signaling act together to regulate coordinated cytoskeletal change in response to even a single guidance cue. Second, it is not clear if a common molecular mechanism is involved in mediating the response to different inhibitory guidance cues. These are difficult problems to address, but the progress to date is promising, and the papers by Winberg, Hu, and colleagues represent important advances toward these goals.

Kristin L. Whitford and Anirvan Ghosh
Department of Neuroscience
Johns Hopkins University School of Medicine
Baltimore, Maryland 21205

Selected Reading

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Unwebbing the Presynaptic Web

The release of neurotransmitter from nerve terminals occurs at a specialized region of the presynaptic plasma membrane called the active zone. A dense matrix of proteins associated with the active zone, called the presynaptic web, is thought to play a fundamental role in defining these neurotransmitter release sites. In this issue of *Neuron*, Phillips et al. have identified conditions for the biochemical purification of the presynaptic web and show that the web is comprised of proteins involved in the docking, fusion, and recycling of synaptic vesicles.

Synaptic junctions, in particular those formed in the central nervous system, are characterized ultrastructurally by the presence of electron dense thickenings associated with the cytoplasmic faces of both the pre- and postsynaptic plasma membranes. The electron dense nature of these membrane specializations has been attributed to a high concentration of proteinaceous material that is readily visible by electron microscopy (EM) due to its very osmiophilic nature (see Peters et al., 1991). Early EM studies by Gray in the late 1950's suggested that synapses could be categorized either as type 1 or 2 depending upon the amount of electron dense material present at the postsynaptic plasma membrane (see Peters et al., 1991). For example, type 1 synapses exhibit a very pronounced electron dense thickening ($\sim$50 nm thick), referred as the postsynaptic density (PSD). These were subsequently shown to be primarily excitatory glutamatergic synapses. In contrast, type 2 synapses had a rather unremarkable PSD and were associated with inhibitory glycinergic or GABAergic synapses (see Peters et al., 1991). PSDs, viewed in cross-section, appear rather uniform in density and thickness from one edge of the junction to the other (see Peters et al., 1991). However, detailed ultrastructural analyses of purified PSDs have shown that PSDs exhibit a planar array of spherical units embedded in a filamentous lattice (see Matus and Taff-Jones, 1978). The PSD appears to be primarily involved in the clustering of both neurotransmitter receptors at high density and proteins involved in downstream signaling pathways. This concept is supported by extensive molecular, biochemical, cell biological, as well as physiological studies accumu-