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Semaphorin Signaling Unplugged: A Nervy AKAP cAMP(s) Out on Plexin

Minireview

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Semaphorins signal through Plexin receptors to mediate a range of predominantly repulsive effects on axons in the developing nervous system. Semaphorin-directed repulsive turning responses of cultured *Xenopus* neurons can be converted to attraction by manipulating cyclic nucleotide signaling pathways. cAMP- and cGMP-dependent response conversions are observed for many families of guidance cues, but a direct molecular link between guidance receptors and cyclic nucleotides has remained elusive. Recent findings indicate that the *Drosophila* A-kinase anchoring protein (AKAP) Nervy couples Plexin to PKA to modulate Semaphorin repulsion, suggesting a mechanism for the integration of diverse signaling inputs to the growth cone.

Semaphorins and Plexins

Semaphorin (Sema) ligands and Plexin receptors have diverse functions both within and outside of the nervous system. During axon guidance, Semas function predominantly as axonal repellants, although in certain contexts they promote attractive responses. For axon repulsion, Sema signals through a variety of cytoskeletal regulatory proteins, including members of the Rho family of small GTPases, to promote local actin depolymerization. Genetic studies highlight the importance of Semas for many guidance events, including the regulation of axon fasciculation, influencing steering decisions, sorting axons into distinct zones, enforcing waiting periods, and contributing to the specificity of target selection (Pasterkamp and Kolodkin, 2003). Semas and their receptors exhibit tremendous structural diversity and are grouped into several distinct classes (Pasterkamp and Kolodkin, 2003). In *Drosophila*, for example, Plexin A (PlexA) together with its coreceptor Off Track are a receptor for the transmembrane Sema1a, while in vertebrates Plexin A1 and its coreceptors Neuropilin and L1 form a receptor complex for class 3 secreted Semas (Figure 1). Here, I focus on how recent advances in understanding the regulation of PlexA repulsion have established a long-sought molecular link between cyclic nucleotide signaling pathways and axon guidance receptors.

Cyclic Nucleotide Signaling and Axon Guidance

Nearly 10 years ago, Mu Ming Poo and colleagues made the startling discovery that reducing the levels of the cyclic nucleotide cAMP or inhibiting protein kinase A (PKA) in the growth cones of cultured *Xenopus* spinal neurons could convert attraction toward sources of brain-derived neurotrophic factor and acetylcholine into repulsion (Song et al., 1997). Additional studies in the *Xenopus* culture system demonstrated that cyclic nucleotide (cAMP or cGMP)-dependent response conversion could also be observed for other attractive guidance cues, such as Netrin (Ming et al., 1997), as well as a number of repulsive cues, including Semaphorins (Song et al., 1998).

The general picture that emerged from these studies was that high cyclic nucleotide levels favor attraction, while low levels favor repulsion. Moreover, response conversion for some guidance cues is dependent on cAMP levels and extracellular calcium, while response conversion for a nonoverlapping set of guidance cues is dependent on cGMP levels and is calcium independent. There were a number of early hints that the story was not as simple as this; for example, cGMP-dependent switching of Semaphorin repulsion into attraction was blocked by cAMP antagonists (Song et al., 1998), suggesting an interplay between the different cyclic nucleotide signaling pathways. Indeed, recent findings support the model in which the ratio of cAMP and cGMP is critical for determining the polarity of the turning response to Netrin, with high cAMP to cGMP ratios favoring attraction and vice versa (Nishiyama et al., 2003). Despite significant progress in understanding some of the signals that lead to changes in cAMP and cGMP levels and how cyclic nucleotide signaling influences cytoskeletal regulation in growth cones, a direct molecular link between these pathways and axon guidance receptors has remained elusive. Now, an important new study of Sema/Plexin signaling in *Drosophila* has established the first such link (Terman and Kolodkin, 2004).

The Nervy AKAP Links Plexin and PKA

The establishment of neuromuscular connectivity in the developing *Drosophila* embryo has proved to be a powerful system for investigating mechanisms of axon guidance and target selection. PlexA and Sema1a are expressed in many motor neurons, where they regulate axon defasciculation at several distinct choice points (Winberg et al., 1998b; Yu et al., 1998). For example, axons of the intersegmental nerve b (ISNb) normally defasciculate from the main branch of the ISN to innervate several ventral muscles; *plexA* and *sema1a* mutants display specific disruptions in these defasciculation events, resulting in missing innervations or the complete bypass of the ventral muscle field; defects that are consistent with reduced axon-axon repulsion (Figure 2).

To find additional molecules that contribute to Sema1a/PlexA-mediated axon guidance in *Drosophila*, Terman and Kolodkin performed a yeast interaction screen using the conserved intracellular C2 portion of the PlexA cytoplasmic domain as bait (Figure 1). One of the molecules that was identified was Nervy, a *Drosophila* protein with significant homology to a small group of myeloid translocation gene products, proteins recently shown to act as AKAPs by binding to the regulatory subunit of the type II PKA holoenzyme in the cytoplasm of lymphocytes (Fukuyama et al., 2001; Schillace et al., 2002). Distinct AKAPs localize type II PKAs to distinct subcellular regions, facilitating the spatially specific phosphorylation of target proteins in response to local elevations of cAMP (Diviani and Scott, 2001; Feli-

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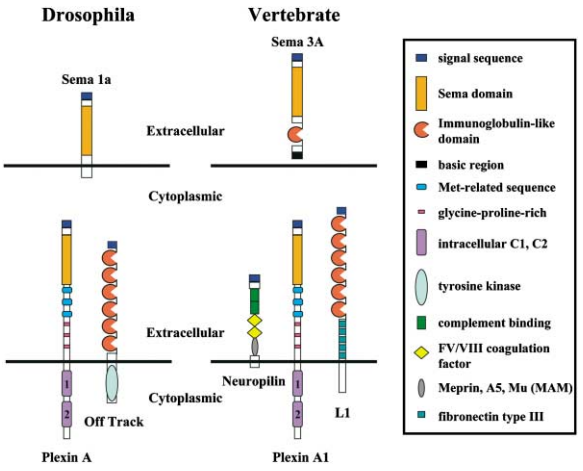

Figure 1. Plexin A Receptor Complexes and the Semaphorins That Are Known to Activate Them in *Drosophila* and Vertebrates Are Diagrammed

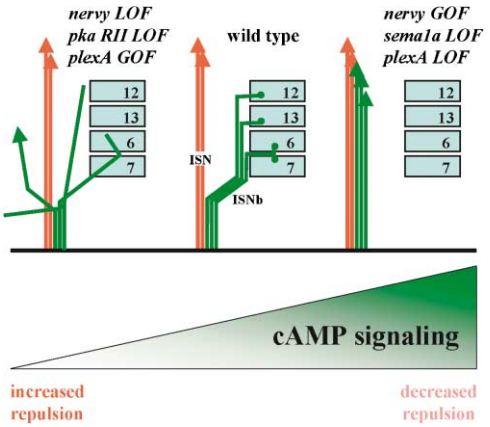

Figure 2. Summary of the Phenotypes Resulting from Perturbations of *sema1a, plexA, nervy*, and *pka RII*

LOF, loss of function; GOF, gain of function. The wild-type innervation of ventral muscles 12, 13, 6, and 7 is shown in the middle panel. For simplicity, only the most severe *sema1a* and *plexA* loss-of-function phenotype, in which ISNb axons completely bypass the ventral muscle field, is diagrammed. The cartoon gradient of cAMP represents the changes in cAMP signaling that are assumed to result from manipulating PKA and Nervy function. Loss of *nervy* or *pka RII* leads to decreased cAMP signaling, increased repulsion, and excessive defasciculation. *nervy* gain of function leads to increased cAMP signaling, decreased repulsion, and excessive fasciculation.

ciello et al., 2001). In addition to localizing PKA, AKAPs, large multidomain proteins, also serve as platforms for the recruitment of many other signaling proteins, including several PKA substrates; thus AKAPs, such as Nervy, are uniquely poised to coordinate multiple PKA inputs and outputs (Diviani and Scott, 2001; Feliciello et al., 2001).

To determine if Nervy anchors PKA to the PlexA receptor in developing motor neurons, and if so, what impact this coupling has on Plexin repulsion, an elegant series of biochemical and genetic experiments were performed. Protein expression pattern analysis revealed that, like PlexA, both PKA RII and Nervy are present in embryonic motor neurons and both are robustly detected in embryonic antineural PlexA immunoprecipitates. Strikingly, *nervy* mRNA is detected at high levels within motor axons, suggesting the potential for rapid regulation at the level of local translation. Physical interactions between Nervy and PKA RII in neurons further support the idea that Nervy is a neuronal AKAP that can link PlexA and PKA (Terman and Kolodkin, 2004).

Genetic manipulation of in vivo Nervy and PKA RII levels during motor axon guidance revealed the significance of these biochemical interactions for PlexA-mediated repulsion. Loss of *nervy* or PKA RII (i.e., decreased cAMP signaling) leads to excessive motor axon defasciculation, phenotypes remarkably similar to PlexA gain of function; satisfyingly, neuronal overexpression of Nervy (i.e., increasing cAMP signaling) results in disruptions in axon defasciculation that appear identical to loss of function of either *plexA* or *sema1a* (Terman and Kolodkin, 2004). These loss- and gain-of-function genetic data suggest that *nervy* and *pka RII* function to antagonize PlexA repulsion (Figure 2). A series of dose-dependent genetic interactions, together with the finding that a single amino acid substitution (Nervy^{V523P}) that prevents Nervy and PKA RII association generates a dominant-negative Nervy, beautifully support the model that, by linking PKA to Plexin, Nervy negatively regulates the repulsive response to Sema1a (Terman and Kolodkin, 2004).

Potential PKA Targets in the Vicinity of Plexin

How does PKA activation near the PlexA receptor antagonize repulsion? In considering likely PKA targets, it is worthwhile to review some of the molecules known to contribute to Sema-mediated repulsion. Semaphorin signaling mechanisms have been extensively studied, and a detailed consideration of all of the known players is well beyond the scope of this minireview. For in-depth surveys of Sema/Plexin signaling, see Castellani and Rougon (2002), Liu and Strittmatter (2001), Pasterkamp and Kolodkin (2003), and the references therein.

Rho family small GTPases and their effectors function downstream of many Plexins to direct cytoskeletal rearrangements (Liu and Strittmatter, 2001). Plexin B repulsion, for example, is mediated in part through the simultaneous inhibition of Rac and activation of Rho (Pasterkamp and Kolodkin, 2003). A direct role for Rho family GTPases in vertebrate PlexA1 repulsion has also been observed; however, in this case the relationship between Rac and Rho activity in mediating repulsion appears to be more complex. PKA is known to phosphorylate and inhibit Rho activity (Dong et al., 1998), suggesting that Rho may be a target of Plexin-associated PKA. Additional potential targets of PKA include the collapsin response mediator proteins (CRMPs), a family of cytosolic phosphoproteins that may link vertebrate Sema3A signals to the Rho GTPases (Liu and Strittmatter, 2001).

Molecule interacting with casL (MICAL), a member of a family of flavoprotein monoxygenases (FMs) with several consensus PKA phosphorylation sites, is another interesting candidate target of PKA for the inhibition of PlexA repulsion (Terman et al., 2002). *Drosophila* MICAL, like Nervy, binds directly to the PlexA cytoplasmic domain, and genetic analysis reveals an essential role for MICAL in Sema1a/PlexA repulsion. Fur-

thermore, specific FM inhibitors neutralize Sema3A repulsion, suggesting a conserved role for MICAL proteins in PlexA repulsion (Terman et al., 2002). The growth cone substrates of MICAL enzymatic activity are not known, although it is interesting to note that redox regulation can modulate signaling protein activities and that oxidation of actin is known to promote depolymerization (Pasterkamp and Kolodkin, 2003). Since both Nervy and MICAL bind directly to the PlexA cytoplasmic domain, one possibility is that Nervy could prevent MICAL function (thereby antagonizing Sema1a/PlexA repulsion) by competing for binding to PlexA. However, the fact that expression of the Nervy^{V523P} dominant-negative (which still binds PlexA but cannot recruit PKA) leads to a phenotype similar to MICAL or PlexA gain of function argues against the idea that Nervy inhibits repulsion through binding competition with MICAL or any other PlexA downstream effectors (Terman and Kolodkin, 2004).

In many cases where an AKAP links PKA to membrane receptors or channels, the transmembrane molecule itself has proven to be a PKA substrate (Feliciello et al., 2001), suggesting that the PlexA receptor could be directly inhibited by PKA phosphorylation. In this regard, it is intriguing to note the presence of a PKA consensus phosphorylation site in the PlexA receptor (Winberg et al., 1998b). One straightforward mechanism by which the PlexA receptor could be directly inhibited would be to downregulate the level of PlexA on the surface of the growth cone. Indeed, Commissureless-dependent control of the surface expression of Roundabout (Robo) receptors has been shown to be a potent mechanism for inhibiting Robo repulsion (Keleman et al., 2002). To date, it has not been possible to directly monitor the surface expression of Plexin receptors to determine if such a regulatory mechanism could exist. Indirect evidence opposing the idea of receptor-level regulation comes from the observation that nerve growth factor (NGF) inhibition of Sema3A repulsion, a PKA-dependent phenomenon, does not affect the surface levels of the Neuropilin coreceptor (Dontchev and Letourneau, 2002). Discovering the relationship between PlexA-associated PKA and any of these putative targets of regulation will surely lead to fascinating insights into the mechanisms of signal integration during axon guidance.

Regulation of Plexin-Associated PKA Activity

The fact that *nervy* mutations result in excessive and premature axon defasciculation suggests that during normal motor axon guidance Sema1a/PlexA repulsion must be actively inhibited and that this is likely achieved through a Nervy/PKA-dependent mechanism. Since basal cAMP levels are insufficient to dissociate and activate the catalytic subunits of anchored PKA (Feliciello et al., 2001), other signaling inputs that lead to local elevations of cAMP are likely required to activate PKA in order to antagonize Plexin repulsion. What signals might control Plexin-associated PKA activity and modulate responses to Semaphorins? Signaling pathways activated by Netrins, Neurotrophins, and G protein-coupled receptors are all good candidates to lead to local elevations in cAMP signaling, and all of these pathways have been strongly implicated in regulating Semaphorin responses (Chalasani et al., 2003; Dontchev and Letourneau, 2002; Winberg et al., 1998a).

Netrin signaling has been shown to lead to elevations

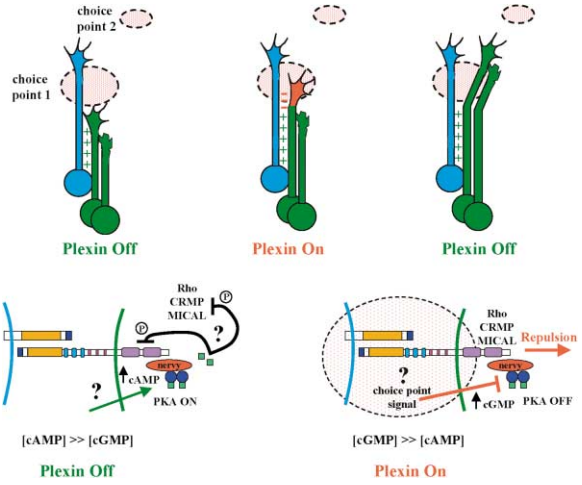

Figure 3. A Model for Nervy/PKA Function at ISN Defasciculation Choice Points

(Top) Before arriving at the first choice point, Plexin repulsion is kept off (green) and axon fasciculation prevails. When the growth cone arrives at the choice point, Plexin is turned on (red), resulting in defasciculation. After leaving choice point one, Plexin repulsion is turned off to prevent premature defasciculation prior to reaching choice point two. The follower green growth cone is represented as less complex in structure than the pioneer. (Bottom) A cartoon representing the molecular events associated with the on and off states of Plexin repulsion is shown. Before the choice point (bottom left), Nervy (red oval) tethered PKA is activated by high cAMP levels (triggered by unknown signals). Activated PKA catalytic domains (green squares) inhibit Plexin repulsion by phosphorylating either Plexin itself and/or Plexin signaling components, such as Rho, CRMP, or MICAL. In the off state, the ratio of cAMP to cGMP is high. At the choice point (bottom right), unknown signals inhibit PKA, potentially by triggering increased local cGMP levels leading to a high cGMP to cAMP ratio. In the absence of PKA inhibition, Plexin repulsion is activated. Signal termination upon leaving the choice point could be achieved by renewed elevation of cAMP triggered by chemoattractants.

of cAMP (Corset et al., 2000), and given the strong genetic evidence of mutual antagonism between Netrin and Sema2 during motor axon guidance and target selection in *Drosophila* (Winberg et al., 1998a), it is tempting to speculate that Netrin signaling could function in certain contexts to regulate Plexin-coupled PKA. NGF activates a broad range of signaling pathways, including the cAMP pathway, and is coexpressed with Sema3A in specific areas of the spinal cord where it could influence Sema responses (Dontchev and Letourneau, 2002). Furthermore, inhibitory modulation of Sema3A-mediated growth cone collapse by NGF in vitro is dependent on increased cAMP levels and PKA activation (Dontchev and Letourneau, 2002). Seven-transmembrane G protein-coupled receptors, a huge receptor family, many of which signal through cyclic nucleotides, are another group of signals known to regulate axon guidance (Xiang et al., 2002) that could impinge upon PlexA-associated PKA. Indeed, recent findings indicate that stromal cell-derived factor 1 (SDF-1), a chemokine that signals through the CXCR4 G protein-coupled receptor, antagonizes Sema3A repulsion in vitro via a cAMP/PKA pathway (Chalasani et al., 2003). In addition, CXCR4 knockout mice show a number of axon guidance defects, including premature entry of subsets of sensory axons

into the dorsal spinal cord, a phenotype consistent with an in vivo role of SDF-1 in modulating Sema3A repulsion (Chalasani et al., 2003).

At least two distinct modes of guidance receptor regulation by anchored PKA that are likely to be employed in different developmental contexts can be envisioned. First, in cases where continuous repulsion is desirable (e.g., axons growing through a channel surrounded by repellants), PlexA-associated PKA would remain inactive; selective activation of PKA later in the axon's trajectory could then shut off repulsion, allowing axons to change their behavior. Alternatively, as is likely the case during ISN/ISNb motor axon guidance, PlexA-associated PKA could function constitutively to dampen repulsion, with transient repression of PKA inhibition, or the influence of an independent signal (e.g., at a defasciculation choice point) leading to a precisely localized burst of repulsion (Figure 3). After axon divergence at specific ISNb choice points, Nervy tethered PKA would be ideally poised to rapidly terminate the PlexA repulsive signal in response to Netrin or other attractant-triggered cAMP elevation, thereby preventing premature defasciculation prior to reaching the next choice point (Figure 3).

The Role of Cyclic Nucleotides: Switching the Sign or Setting the Gain?

In addition to the cGMP-dependent response conversion of Sema repulsion in cultured *Xenopus* neurons, more recent evidence suggests that the extracellular domain of L1 presented in *trans* can also lead to a cGMP-dependent response conversion of Sema3A repulsion in cultured cortical neurons (Castellani et al., 2002). In contrast, there is accumulating evidence that cAMP can also regulate Semaphorin responses, but in the case of cAMP, the effect appears to be to repress the repulsive response rather than to convert it to attraction (Chalasani et al., 2003; Dontchev and Letourneau, 2002; Terman and Kolodkin, 2004). For example, SDF-1 and NGF both antagonize the growth cone collapse induced by Sema3A through activation of the cAMP signaling pathway (Chalasani et al., 2003; Dontchev and Letourneau, 2002). In these cases, Sema3A still acts as a repellant, but its potency is greatly diminished. In neither of these studies is it clear how the effects of PKA are directly coupled to the PlexA1 receptor, although it is tempting to speculate by analogy to Nervy that there is an unidentified AKAP involved. The *nervy* gain-of-function phenotype also supports the idea that cAMP signaling dampens PlexA repulsion rather than converting it to attraction, lending in vivo support to the idea that cAMP can modulate guidance responses (Terman and Kolodkin, 2004). Together, these three studies define an additional role for cyclic nucleotide regulation of axon guidance responses, which appears to be distinct from response conversion.

The inhibition of repulsion mediated by cAMP signaling appears at odds with observations from the *Xenopus* turning assays, where cAMP had no striking effect on Semaphorin responses, though it was required for the cGMP-induced response conversion (Song et al., 1998). Recent findings that the ratio of cAMP and cGMP rather than the absolute levels of the individual cyclic nucleotides is paramount in determining the growth cone response may help explain these differences (Nishiyama et al., 2003), since studies have not yet addressed the coordinate regulation of cAMP and cGMP signaling in the context of PlexA repulsion. Alternatively, the different effects of cAMP may reflect the different cellular contexts in which these pathways are activated. Future studies in which cyclic nucleotide signaling pathways are simultaneously manipulated and measured promise to provide further insight into the regulation of Sema repulsion. Of particular interest will be determining how cAMP and cGMP pathways are coordinated in vivo and how the effects of cGMP signaling are spatially controlled. Are there GKAPs lurking undetected on guidance receptors, or could spatial control be conferred through complexes between guidance receptors and receptor guanylyl cyclases? Given the complexity of wiring the nervous system, the answer to the question "do cyclic nucleotides change the sign or modulate the strength of axon guidance responses?" will undoubtedly prove to be "both."

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