To Cross or Not to Cross: Alternatively Spliced Forms of the Robo3 Receptor Regulate Discrete Steps in Axonal Midline Crossing

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How growth cones grow toward and then away from intermediate targets is a key issue in axon guidance. In this issue of *Neuron*, Tessier-Lavigne and colleagues demonstrate that two different spliced isoforms of the Robo3 receptor act sequentially in commissural neuron growth cones to mediate this process at the ventral midline in the vertebrate spinal cord.

Growth cones at the leading edge of developing axons often migrate over long distances to make synaptic connections with target cells (Yu and Bargmann, 2001). They typically travel along stereotyped pathways, migrating in stepwise fashion between intermediate targets or ''guidepost'' cells. Growth cones must first be attracted to these targets and subsequently migrate away from them. The precise spatiotemporal expression of attractants and repellents and the sensitivity of growth cones to these signals play crucial roles in regulating the dynamic behavior of growth cones in the developing embryo.

The most extensively studied and well-understood step in axon guidance is the sequential growth of spinal commissural neurons toward, across, and away from the ventral midline (Garbe and Bashaw, 2004). Netrin is the key attractant, which is secreted by a specialized group of cells in the ventral midline called the floor plate. Contact-dependent mechanisms promote extension of growth cones across the floor plate to the contralateral side, whereupon growth cones acquire sensitivity to the midline repellent Slit and grow away from the midline. This response to Slit is mediated by the receptor Roundabout (Robo), which is transcribed in commissural neurons both before and after crossing the midline. Studies in both vertebrates and invertebrates support the notion that inhibitory mechanisms for Robo act within commissural growth cones to prevent their premature response to Slit, thus allowing them to progress toward and across the midline (Dickson and Gilestro, 2006). In flies, a multipass transmembrane protein called Commissureless is expressed in commissural neurons prior to crossing the midline and antagonizes Robo function by preventing accumulation of Robo in growth cones (Keleman et al., 2002). Similarly, the Tessier-Lavigne lab demonstrated that a Robo homolog in mouse called Robo3 is expressed at high levels in the developing commissural neurons prior to midline crossing and prevents precocious activation of Robo1 and Robo2 (Sabatier et al., 2004). This ensures that the Slit-dependent repulsive signal is not detected by commissural neurons until their growth cones cross to the contralateral side. While the function of Robo3 is formally similar to Commissureless, how Robo3 acts at a mechanistic level is poorly understood.

As Tessier-Lavigne and colleagues now report (Chen et al., 2008 [this issue of *Neuron*]), Robo3 regulates midline crossing in a more complex fashion through sequential utilization of two different Robo3 isoforms generated through alternative splicing. cDNA sequencing and immunohistological studies revealed that these two forms differ in the excision or retention of one intron in the pre-mRNA, leading to mRNAs that encode different C-terminal cytoplasmic signaling domains. The Robo3.1 product accumulates in commissural neurons on the ipsilateral side, while Robo3.2 protein is markedly upregulated only contralaterally. Robo3.1 prevents precocious midline repulsion by antagonizing the function of Robo1 and -2, either directly or indirectly, prior to midline crossing. By contrast, the Robo3.2 isoform, like Robo1 and -2, promotes repulsion away from the midline once growth cones have crossed to the contralateral side.

These studies provide an example of a binary switch in growth cone signaling mediated by two alternatively spliced isoforms of a single receptor. However, as discussed in the paper, the regulation of this switch in protein expression may not be at the level of splicing. While Robo3.1 and Robo3.2 proteins are clearly expressed in different spatiotemporal domains, Robo3.1 before midline crossing and Robo3.2 after, the relative amounts of the two mRNAs remain constant over this developmental timeframe. Thus, the differential control of Robo isoform expression likely lies downstream from mRNA splicing.

The differential expression of Robo3.1 and Robo3.2 could occur through differential mRNA localization, translation, or protein stability or some combination of these processes. Robo3.1 and Robo3.2 could be differentially targeted to the protein degradation machinery before and after midline crossing. This would require spatial regulation of proteins that allow the selective ubiquitination of Robo3.1

and Robo3.2 outside their appropriate domains. Alternatively, the mRNA for Robo3.1 might itself be specifically enriched in precrossing axons, while the Robo3.2 mRNA could be transported out to the contralateral process only after crossing. This mechanism would require the silencing of the Robo3.2 mRNA prior to midline crossing. Finally, the translation of the Robo3.1 and Robo3.2 mRNAs could change as the axon crosses the midline; for instance, Robo3.2 translation could be repressed prior to crossing, only to be relieved by intercellular signaling events modulating translation in growth cones as they transit to the contralateral side of the midline.

While the mechanisms regulating the differential accumulation of these two Robo3 isoforms remains unclear, the structure of Robo3.2 suggests that this transcript may be under unusual translational control through the nonsense-mediated decay pathway (Chang et al., 2007). Typically, stop codons that are not found in the 3′-most exon will induce NMD, thereby preventing the expression of genes carrying nonsense or frameshift mutations whose production of a truncated protein might be deleterious. Recently, the NMD pathway has been shown to regulate the expression of many naturally occurring splice variants as a normal mechanism of downregulation. Robo3.2 has the hallmarks of such a natural NMD target. The alternative splicing pattern exhibited by Robo3 is called intron retention (Li et al., 2007). In Robo3.2, the intron between exons 26 and 27 is retained, while the Robo3.1-encoding transcript is fully spliced. As a consequence, the stop codon in Robo3.2 is in the retained intron upstream of the exon 27/exon28 splice junction and thus predicted to induce NMD. By contrast, the stop codon in Robo3.1 is in the final exon and should not be subject to NMD. Thus, NMD could cause downregulation of the Robo3.2 message in commissural neurons on the ipsilateral side and thus the predominance of Robo3.1 protein in these axons.

NMD is mediated by the exon junction complex (EJC), which is also an important regulator of translation (Le Hir and Séraphin, 2008). The EJC is an assembly of proteins that is deposited, in a non-sequence-specific manner, onto the RNA upstream from each exon/exon junction during the process of splicing. The EJC contains several core proteins, including eIF4A3, and has a number of important effects on mRNA expression, including stimulation of translation of mRNAs from intron-containing genes. After deposition of the EJCs during splicing and export of the final mRNA to the cytoplasm, it is thought that the EJCs are stripped from the mRNA during the initial or pioneer round of translation. If translation terminates more than 50 nucleotides upstream of an EJC, the complex will recruit the NMD factors UPF1, -2, and -3, leading to degradation of the RNA. The EJC and its components are also known to contribute to localized translation, as seen with the Oskar protein during *Drosophila* embryogenesis (Hachet and Ephrussi, 2004).

A recent study suggests that signaling can modulate the expression of NMD targets in the mature CNS (Giorgi et al., 2007). Arc mRNA is dendritically localized and translated at synapses, where it can be induced by brain-derived neurotrophic factor (BDNF) and contributes to synaptic strengthening during long-term potentiation (LTP). Arc mRNA is also a natural NMD target. The Arc gene contains two introns within its 3′ UTR, and the eIF4A3 protein was shown to colocalize with Arc mRNA in dendrites. Moreover, knockdown of eIF4A3 leads to increased Arc expression at synapses. Thus, one model for Arc upregulation in response to LTP or BDNF stimulation is that it reflects a signal-dependent inactivation of eIF4A3.

The upstream stop codon of Robo3.2 predicts that it could be subject to similar modes of control as Arc, except that, rather than in mature cells in response to excitation, the Robo3.2 regulation occurs in developing axons in response to cues received during midline crossing. The EJC and NMD components could play a crucial role in downregulating Robo3.2 mRNA in commissural neurons on the ipsilateral side of the midline. Upon interaction with signals at the midline, these components may be inactivated to allow expression of Robo3.2 and growth of these neurons away from the midline on the contralateral side. While this provides a plausible explanation for the selective expression of Robo3.1 before commissural neurons reach the midline, it does not explain the downregulation of Robo3.1 in axonal segments on the contralateral side. It will be very interesting to examine the expression of EJC components in extending axons and to test the effect of EJC and UPF protein knockdown on Robo3.2 expression.

In conclusion, studies of midline guidance continue to provide important insights into the molecular mechanisms by which growth cone responses to guidance signals are dynamically regulated at intermediate targets. While core components of the pathways, the Robos, Slits, Netrins, and Netrin receptors are evolutionarily conserved, a diverse set of mechanisms have evolved to regulate their expression in an instructive fashion in different organisms.

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