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Retinal axon guidance: novel mechanisms for steering

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Abstract

Axons from the retina traverse different molecular territories as they navigate to the tectum. A single territory might span only a few cell diameters and harbour multiple guidance cues, many of which are beginning to be characterized. Also present in the pathway are ‘modulators’ that influence a growth cone’s response to a coincident signal but do not guide growth directly. An emerging principle is that the growth cone, itself, changes molecularly as it journeys through the visual pathway. Growing retinal axons contain mRNAs, ubiquitinating and apoptotic enzymes, translation and degradation machinery. Guidance cues can trigger rapid and local synthesis, degradation and endocytosis of proteins, providing a fast and flexible way for growth cones to respond to cues in their microenvironment and to alter their responsiveness. The data raise the idea that the localized synthesis and downregulation of proteins might help to steer retinal axon growth and, further, might contribute to the changing character of a growth cone as it ages.

Introduction

The vertebrate visual system is one of the best-studied model systems, with regard to axon guidance and topographic mapping. The study of retinal ganglion cell (RGC) axons, as they navigate from the retina to the tectum, continues to provide fundamental insights into the mechanisms involved in growth cone steering. The visual pathway in lower vertebrates can be divided into short, molecularly distinct segments, beginning with the retinal surface. Here, regardless of the point of topographic origin in the retina, axons from RGCs grow in a directed manner towards the optic nerve head (ONH), through which they exit the eye to enter the optic nerve. The optic nerve joins the ventral diencephalon and contralateral-projecting axons cross the midline at the optic chiasm, then grows dorsalwards, through the diencephalons, thus, forming the optic tract. From the dorsal optic tract, axons enter the optic tectum (superior colliculus in mammals), their main synaptic target in the midbrain, where they terminate in a topographic array. Guidance cues, such as netrins, slits, semaphorins and A- and B-type ephrins decorate different segments of the pathway, providing guidance signals that RGCs detect via appropriate receptors (Figure 1; [1–3]).

Research over the past few years has identified novel guidance molecules, some of which act as conventional ‘signposts’ by directly steering axon growth, and others that fall into a class of ‘modulators’ that act to enhance or diminish responses to signpost cues. It has become clear that a single guidance molecule can act as an attractant or a repellent, depending on the extracellular and intracellular context of the growth cones. It has also become clear that growth cones change their responsiveness to certain guidance cues as a function of their age. Finally, growth cones have been found to dynamically change their constituent proteins in response to various environmental signals, which suggests that their responsiveness might be a function of their stimulus history. Experiments on RGC axons

have significantly improved our understanding in all of these areas. Other exciting advances in the field of retinal axon guidance are the elucidation of the way that axons choose to grow ipsilaterally or contralaterally at the optic chiasm ([4–7] and Mason and colleagues, this issue), how they stay confined to the optic pathway [8–11] and the mechanisms that underlie topographic mapping along the dorso-ventral (medio-lateral) dimension in the tectum [12,13]. The current review focuses primarily on new concepts and molecular mechanisms that are involved in axon guidance, such as crosstalk modulation, age-related intrinsic changes, local translation and termination of signaling through degradation and endocytosis.

Signaling crosstalk and ‘modulators’

Multiple signals are likely to impinge simultaneously on a growth cone *in vivo*, and each one can differentially affect intracellular levels of cyclic nucleotides. Secreted axon guidance molecules were originally grouped into two main classes, according to their dependence on either cAMP (cyclic adenosine monophosphate; group I) or cGMP (cyclic guanosine monophosphate; group II) signaling for growth cone turning [14]. This simple classification has been revised recently, because a reinvestigation has shown that it is the ratio of cAMP:cGMP that is key in determining the direction of growth cone turning to netrin-1 [15] — a high ratio of cAMP:cGMP favours attraction, whereas, a low ratio favours repulsion. These findings implicate the likely importance of crosstalk modulation between different guidance signaling pathways. Indeed, at the ONH, interaction between laminin-1 and netrin-1 signaling pathways, via cAMP, is thought to play an important role in guiding retinal axons out of the eye [16].

Along similar lines, the chemokine stromal cell derived factor-1 (SDF-1), has been newly identified as a modulator of multiple axonal repellents. SDF-1 reduces the repellent activity of Slit-2 on retinal growth cones [17]. This is a modulatory effect because SDF-1 has no detectable attractant or repellent activity on RGC axons by itself when used in a collagen gel explant assay. Although, the possibility remains that SDF-1 might induce directional turning in RGC growth cones if tested in a short, one-hour gradient assay, instead of the long (20-hour) collagen explant assays. SDF-1-modulated Slit-2 responsiveness is mediated through a G-protein coupled receptor, CXCR4, whose activation stimulates an elevation of cAMP. SDF-1 also decreases the repellent activities of the semaphorins Sema3A and 3C in other neurons and CXCR4-mutant mice have axon guidance defects in the spinal cord, suggesting a general role of SDF-1 in modulating axonal responsiveness to various guidance cues. SDF-1 is a known chemoattractant for leukocytes, dentate granule cells and cerebellar neurons [18,19], and cultured rat cerebellar axons are repelled by a gradient of SDF-1, indicating that SDF-1 can also act as guidance cue by itself and that its effects might be highly cell type-specific [20].

In the fly visual system also, an axon guidance modulator has been identified in the form of the hormone insulin [21]. Insulin signals through its insulin receptor (InR) and InR-mutant *Drosophila* show diverse axon-targeting errors of photoreceptor cell axons in the medulla. Like chemokines, insulin circulates widely; cells along the visual pathway do not secrete insulin, thus, suggesting that insulin is not, itself, a directional cue [22]. It is likely that multiple modulators exist in the developing nervous system — future studies will face the challenge of addressing the way that the growth cone interprets the cyclic nucleotide changes to produce an appropriate response.

Local protein synthesis in retinal growth cones

The prevailing view, until two years ago, was that axons do not synthesize proteins. This view has been over-turned recently, by the demonstration that axons, including growing retinal axons and growth cones, contain ribosomes, mRNA and translation initiation

proteins, and can synthesize proteins [23^{**},24,25^{**},26]. A key finding is that axon guidance molecules, like netrin-1 and semaphorins, trigger protein synthesis in retinal growth cones within 5–10 min of addition in the absence of their cell bodies. Inhibition of protein synthesis with translation blockers abolishes the chemotropic responses of growth cones to netrin-1 and Sema3A, demonstrating that fast, local synthesis is essential for cue-directed guidance *in vitro* [25^{**}]. Growth cone translation in retinal neurons is rapamycin-sensitive and is, therefore, mTOR- and cap-dependent, and requires MAPK (mitogen activated protein kinase) p42/44 [25^{**},27^{*}]. Although netrin-1 and Sema3A converge on this common pathway, netrin-1-stimulated translation is sensitive to inhibitors of MAPKp38 and PI3 (phosphoinositide 3) kinase, whereas, Sema3A is not, thus, illustrating that two guidance cues initiate translation via divergent signaling cascades. Studies are currently in progress to identify the mRNAs in retinal growth cones. The mRNAs identified, so far, include cytoskeletal, cytoskeletal-related proteins (e.g. β -actin, profilin and cofilin; [23^{**},28]) and cell surface molecules (NCAM [neural cell adhesion molecule], EphA2; [23^{**}]).

Commissural neurons can synthesize and insert new receptors in their growth cones after crossing the netrin-1-rich floor plate (an intermediate target), supporting the idea that environmental signals can trigger lasting changes in growth cones that lead to altered behaviour [23^{**}]. This type of RNA-based mechanism has been proposed as an attractive explanation for the finding that commissural growth cones lose their attraction to netrin-1 after crossing the floor plate [29]. Translation has also been implicated in the adaptation response of *Xenopus* spinal growth cones to gradients of guidance cues [30]. The key questions in all of these studies are: which proteins are translated in response to various axon guidance cues and how does their translation control the chemotactic turning behaviour of a growth cone? The answers to these questions might shed light on the underlying reasons for the stimulation of protein synthesis by guidance cues. One model centres on the idea that restricted contact with a guidance cue, for example, through a single filopodium, elicits fast and localized changes in protein levels (synthesis and/or degradation) in a compartment of the growth cone, leading to asymmetric cytoskeletal rearrangements and turning (Figure 2a). Compartmentalized changes of this type appear to be key in the activity-driven synthesis and degradation of proteins in dendrites [31].

Signaling through destruction: degradation, cleavage and endocytosis

Numerous cellular processes are coordinately regulated by protein synthesis and degradation [31]. This also seems to be the case for the chemotropic responses of growth cones. Retinal growth cones contain the machinery for proteasomal degradation (proteasome proteins, ubiquitin and ubiquitinating enzymes) and their chemotropic responses to guidance cues, such as netrin-1, EphB and lysophosphatidic acid (LPA) but not Sema3A, are blocked by inhibitors of proteasome function [25^{**},32^{*}]. Like translation, guidance cues stimulate the degradation pathway on a rapid timescale, as revealed by a rise in ubiquitin-conjugated proteins within five minutes. Another 'destructive' molecule, caspase-3, usually associated with the apoptotic pathway, has been found to be involved in chemotropic guidance in retinal axons [27^{*}]. Netrin-1 and LPA both cause the activation of caspase-3 in retinal growth cones in a p38- and proteasomal-dependent manner, and inhibition of caspase-3 with a specific peptide inhibitor prevents turning and collapse [27^{*}]. It is likely that activation of caspase-3 in a confined cellular compartment of the growth cone does not activate the full apoptotic cascade but, rather, leads to the confined destruction of specific proteins. One of the known cleavage targets of caspase-3 is the translation initiation factor, eIF4G [33,34], and this raises the possibility that guidance cues, such as netrin-1 and brain derived neurotrophic factor, which simultaneously activate both translation and caspase pathways, negatively regulate the synthesis of proteins that they stimulate.

Another destructive mechanism that operates in growth cones is the removal and/or inactivation of activated receptors. Growth cones have to disengage from surfaces to continue advancing, which means that initial adhesive contacts must be broken. There are several ways to achieve this, including proteolytic cleavage [35], transreceptor silencing [36] and endocytosis. A recent study *in vivo* showed that metalloproteases regulate the growth and guidance of RGC axons. Application of inhibitors of metalloproteases in the *Xenopus* exposed brain preparation resulted in pathfinding and target recognition errors [37]. In this study, the targets of proteolysis are unknown but axon guidance cues, receptors, as already shown for DCC (deleted in colorectal cancer) [38], and adhesion molecules can be cleaved by metalloproteases, thus, underscoring the importance of these enzymes in axon guidance [39].

Endocytosis of functional ephrinB/EphB complexes is sufficient to promote cell detachment of the interacting cells *in vitro* and contact-induced ephrinB–EphB signaling complexes can be endocytosed bidirectionally into the ligand-expressing, as well as in the receptor-expressing, cells. [40,41]. In RGC growth cones, soluble EphB ectodomains cause rapid collapse and are rapidly internalized by endocytosis [32]; this process is functionally important because blocking EphB2 internalization inhibits growth cone collapse [32]. These results suggest that endocytosis provides a fast mechanism for terminating adhesive contacts between growth cones and neighboring cells, and also indicate that the internalized receptor complex activates cytoskeletal changes that are required for retraction/repulsion (Figure 2b). EphrinA and Sema3A were also shown to induce endocytosis [42,43], suggesting that endocytosis might be a general signaling mechanism.

Intrinsic changes in growing axons

For retinal growth cones to move beyond an attractive region of the pathway, such as the ONH, they will probably need to switch off one attraction and gain a new one. Retinal axons first meet netrin-1 at the beginning of the optic pathway, in the ONH, and are attracted to grow into this region. Later, when they have extended some 400 μm further along the pathway, they encounter netrin-1 again, in the optic tract, but appear to ignore or avoid it. This raises the possibility that ‘experience’ alters the behaviour of growth cones. Indeed, studies in the spinal cord have shown that commissural axons lose their attraction to netrin-1 after crossing the floor plate [29] and retinal axons gain responsiveness to Sema3A, only after entering the distal optic pathway [44]. Using a whole visual pathway preparation in *Xenopus* and testing the turning responses of retinal growth cones that had reached progressively more distal points in the pathway, it was shown that growth cones gradually change their responsiveness to netrin-1 from attraction to repulsion as they navigate from the retina to the tectum [45]. This result supports the concept of an experience-based mechanism that is responsible for the changes, but it does not eliminate the possibility that an intrinsic, age-based mechanism is also involved. When this was investigated with retinal neurons, aged in culture, without pathway experience, strikingly, it was found that pathway-naïve axons underwent a similar shift in responsiveness. This shift in behaviour was found to correlate with a decline in levels of intracellular cAMP and netrin-1 receptor (DCC and [adenosine 2b receptor] A2b) expression. These findings indicate that developing retinal neurons have an intrinsic program of change that contributes to a growth cone’s changing sensitivity and suggest the existence of an intrinsic clock or timer mechanism (Figure 2c). What is the basis of such a clock? An interesting answer, in the light of recent findings on local translation and degradation, might be that the composition of mRNAs in growth cones changes over time, enabling the synthesis of new and the degradation of old signaling or receptor molecules.

Conclusions

Over the past few decades, much progress has been made in identifying the molecular and cellular mechanisms that regulate RGC axon guidance in the retinotectal system. RGC guidance is dependent on the presence of guidance cues and modulators, the age and experience of the axon, and also processes such as protein synthesis/degradation and endocytosis. Together, they provide the basis for rapid local growth cone responses in discrete regions of the retinotectal pathway. Much of the evidence for the mechanisms discussed here is taken from studies of retinal axons *in vitro*. The challenge for the future will be to test whether these processes play an important role in axon guidance *in vivo* and to explain how growth cones interpret multiple, often opposing signals, at choice points. To this end, it will be important to elucidate the spatiotemporal and hierarchical regulation of interacting signaling pathways.

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Abbreviations

cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
DCC	deleted in colorectal cancer
LPA	lysophosphatidic acid
MAPK	mitogen activated protein kinase
ONH	optic nerve head
RGC	retinal ganglion cell
SDF-1	stromal cell derived factor 1

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Figure 1.

Diagram of the embryonic visual pathway. Guidance molecules belonging to the netrin, slit, semaphorin and ephrin families are expressed in multiple places along the pathway, in discrete segments, and serve to direct the growth of RGC growth cones. For simplicity, the positions of only a few cues are shown. ONH, optic nerve head; RGC, retinal ganglion cell.

Figure 2.

Models for control of growth cone steering. **(a)** Axon guidance cues, such as Sema3A, netrin and LPA (yellow spots), can trigger local protein synthesis and degradation, which directs growth cone steering. **(b)** Axon guidance cue and receptor complexes can be endocytosed upon cell–cell contact, leading to detachment and withdrawal of the growth cone. A key component of these two models is that the triggered changes are localized asymmetrically to a sub-compartment of the growth cone. **(c)** Growth cones change their responsiveness with age, correlating with a decrease in cAMP levels. The changing internal character of the growth cone as a result of age and experience is indicated by a change in colour.