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Axon Guidance: Growth Cones Make an Unexpected Turn

Dispatch

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Axonal growth cones can turn in response to minute concentration differences in extracellular guidance cues. Surprising new work suggests that these cues might steer the growth cone by inducing rapid local changes in protein levels.

Neuronal growth cones are fascinating structures. Ramon y Cajal first observed them a century ago, and correctly guessed their function in guiding the axons and dendrites of differentiating neurons towards their targets. Since then, successive generations of neuroscientists have sought to understand how growth cones accomplish this extraordinary feat. In a classic series of experiments in the 1940s and 50s, Roger Sperry [1] showed that growth cones are guided by specific chemical cues, and suggested that they might often be distributed in concentration gradients. In the 1970s and 80s, Friedrich Bonhoeffer and colleagues [2] developed clever *in vitro* assays for growth cone guidance, and used these assays to show that growth cones can detect gradients that differ by as little as 1–2% across their diameter.

More recently, in the mid-late 1990s, a remarkable convergence of biochemical studies in vertebrates and genetic studies in invertebrates led to the identification of four major families of guidance molecules — netrins, Semaphorins, ephrins and Slits – all of which can act as graded cues to guide axonal growth cones [3]. With these extracellular guidance cues identified, and powerful *in vitro* and *in vivo* assays available, attention is now increasingly turning to the inner workings of the growth cone. What exactly happens inside the growth cone to make it turn in response to these extracellular gradients? In a surprising new development, Campell and Holt [4] have demonstrated that these turning responses are dependent upon localized protein synthesis and degradation within the growth cone.

Holt and colleagues, like Sperry and Bonhoeffer before them, study the guidance of retinal ganglion cell axons to their targets in the tectum. In earlier work, they showed that *Xenopus* retinal axons are responsive to both the netrin-1 and Semaphorin-3A (Sema3A) guidance cues [5,6]. Netrin-1 appears to guide these axons at an early stage (stage 24), attracting them to the head of the optic nerve on the first leg of their journey towards the tectum [5]. Only later (after about stage 32) do retinal axons respond to Sema3A, which provides a repulsive signal to help keep them on course as they approach the tectum [6]. The netrin-1 and Sema3A responses can both be reproduced *in vitro*, using a growth cone turning assay initially developed for *Xenopus* spinal axons by Poo and colleagues [7] and adapted for retinal axons in the Holt lab [5]. In this assay, an isolated *Xenopus* axon growing on a coverslip is confronted with a gradient of netrin-1 or Sema3A, delivered from a micropipette positioned just ahead and to the side of the advancing growth cone. Retinal axons turn towards the pipette if it delivers netrin-1, and away from it if it provides Sema3A.

Several years ago, Harris, Holt and Bonhoeffer [8] discovered a remarkable autonomy of retinal axon growth cone guidance. Severed from their soma, 'disembodied' growth cones stay alive for up to 3 hours *in vivo* and continue to navigate correctly towards the tectum. Following up from that study, Campbell and Holt [4] found that the isolated growth cones also stay alive in culture for 2 hours, long enough to perform the turning assay. In these assays, the growth cones of severed axons respond to both netrin-1 and Sema3A in the same way as those of intact neurons. These findings are a compelling demonstration that turning relies solely on those molecules, including any mRNAs, already available in the growth cone.

As anticipated from earlier results [9,10], Campbell and Holt [4] found both mRNA and ribosomes in the growth cones of *Xenopus* retinal axons. Furthermore, when they added either netrin-1 or Sema3A to the culture medium, new protein synthesis occurred, as evidenced by the altered phophorylation state of translational regulators and the incorporation of radiolabelled leucine into new proteins. Both of these responses were rapid, occurring within a few minutes of stimulation. Curiously, rapamycin, an inhibitor of the TOR pathway, blocked protein synthesis induced by either netrin-1 or Sema3A, but inhibitors of phosphatidyl inositol 3-kinase (PI 3-kinase) only blocked protein synthesis induced by Sema3A. This suggests that netrin-1 and Sema3A might act at least in part through different pathways to regulate translation within the growth cone.

Having shown that new proteins are synthesized in the growth cone, Campbell and Holt [4] also wondered if proteins might be degraded. For netrin-1, at least, this appears to be the case. As judged by staining for a number of different antibodies, the machinery for proteasome-mediated protein degradation is also present in the growth cones of *Xenopus* retinal axons, and netrin-1 (but not Sema3A) induces a rapid rise in the levels of proteins tagged with ubiquitin and thus destined for degradation in the proteasome.

Netrin-1 thus induces rapid protein synthesis and degradation within the growth cone, while Sema3A appears to induce only protein synthesis. Is this local protein turnover involved in growth cone turning? To test this, Campbell and Holt [4] applied inhibitors of translation or degradation to both intact and isolated retinal axon growth cones in the *in vitro* turning assay.

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The results were clear, and consistent with biochemical and immunohistochemical data: inhibitors of either protein synthesis or degradation blocked attraction by netrin-1, while only protein synthesis inhibitors blocked repulsion by Sema3A. Importantly, none of these inhibitors blocks axon extension itself – growth cones continued to advance at exactly the same rate, simply growing straight ahead rather than turning towards or away from the pipette.

Like all important discoveries, these results raise many more questions than they answer. First and foremost, one would like to know which proteins are newly made or degraded, and how their turnover contributes to growth cone turning. One candidate for a newly-synthesized protein is β-actin [10,11], though clearly there must be others. Identifying the mRNAs present in the growth cone, and finding out which ones are translated in response to each guidance cue, is a challenging but important task for the future.

Equally important is determining how local protein synthesis and degradation contribute to growth cone turning. One idea, favoured by Campbell and Holt [4], is that the asymmetric synthesis or degradation of proteins within the growth cone directly mediates the turning response. For example, in the case of a netrin-1 gradient, cytoskeletal proteins might be synthesized on the side of the growth cone facing up the gradient, and degraded on the side facing down. This could lead to net growth up the gradient towards the netrin-1 source. This is an appealling model, but alternatives also need to be considered. One idea that might be worth pursuing is that growth cones adjust their protein levels not in order to turn, but rather to maximize their sensitivity to the ligand gradient. Locally adjusting the levels of receptors or other signalling molecules might be necessary to ensure that the growth cone can always detect a subtle difference in relative ligand concentration, even as the absolute concentration changes dramatically as it migrates up or down the gradient.

Taking this idea one step further, protein synthesis and degradation induced by one guidance cue might also increase or decrease the growth cone's sensitivity to other cues. Few axons follow just one cue all the way to their target. More often, a series of distinct cues guide them along each successive leg of their journey. Ensuring that growth cones respond to each of these cues in a precise temporal sequence is critical for accurate pathfinding. The work of Campbell and Holt [4] suggests an interesting way in which this could be achieved. Exposure to each guidance cue in the sequence might induce the synthesis of new proteins required to respond to the next, and perhaps also the degradation of those that mediated the preceding response. *Xenopus* retinal axons would be a good model with which to test this idea.

As exciting as it is, this new work of Campbell and Holt [4] in some ways only adds to our sense of bewilderment. A decade or so ago, the growth cone was something of a 'black box'. Not any more. There is already a growing list of proposed mechanisms for growth cone turning, to which we now must add local protein turnover. The other leading contenders to date include Rho family GTPases [12,13], cyclic nucleotides [14,15], phospholipids [16], and transient bursts of calcium release within the growth cone [17–19] and its filopodia [20]. Can all these diverse mechanisms be united into a coherent explanation of growth cone turning? One view, likely to be favoured by those who acknowledge that life is always more complex than we might wish, is that these are all just small glimpses of a much larger and more complex picture that is only gradually being revealed. Plausible biochemical links can indeed be made between many of these pathways, making this a real, if daunting, possibility. Alternatively, it may be that different growth cones use different mechanisms to respond to different cues, so that the diversity of mechanisms is merely a reflection of the diversity of systems under investigation. Either way, it is going to take quite some effort to sort out all the details.

So is there any hope left for those of us who still cling to the notion that there really should be some simple and general mechanism for growth cone turning? Could just one of these mechanisms, or one still undiscovered, be the primary, direct and universal mediator of growth cone turning? Perhaps it is time to raise the bar a little higher in defining the features we expect of a 'mediator' of growth cone turning. Being required for turning *in vitro* is clearly not enough. Growth cones are, after all, exquisitely sensitive devices, and we should not be too surprised to find so many different ways of throwing them off course. The truly astounding feat, after all, is that growth cones can stay so well on course *in vivo*, and that in some cases this behaviour can even be reproduced *in vitro*.

What then should we be looking for? The most demanding list would include at least the following three criteria for a cytoplasmic 'mediator' of growth cone turning. First, an asymmetry in an extracellular guidance cue should be rapidly translated into an asymmetry in the intracellular mediator, before any overt redistribution of cytoskeletal or membrane components within the growth cone becomes apparent. Second, artificially creating such an internal asymmetry should be sufficient to induce turning, even in the absence of any extracellular cue. Third, preventing the formation of this asymmetry should block growth cone turning, not just *in vitro*, but also *in vivo*. Testing each of these criteria is a tall order, but one that must be fulfilled before we can really claim to know what it is that makes growth cones turn. So far, none of the leading contenders for a primary mediator of growth cone turning satisfies all three criteria. Cyclic nucleotide levels and intracellular calcium transients come closest. For local protein synthesis and degradation, none of these three criteria have been tested. Not yet, at least. Stay tuned.

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