

# Signaling at the Growth Cone: The Scientific Progeny of Cajal Meet in Madrid

## Meeting Report

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*I had the good fortune to behold for the first time that fantastic ending of the growing axon. In my sections of the spinal cord of the three day chick embryo, this ending appeared as a concentration of protoplasm of conical form, endowed with amoeboid movements. It could be compared with a living battering ram, soft and flexible, which advances, pushing aside mechanically the obstacles which it finds in its path, until it reaches the region of its peripheral termination. This curious terminal club, I christened the growth cone.*

*Santiago Ramón y Cajal, Recollections of My Life*

In October 2001, over fifty scientists and students from around the world met in Madrid to present their most recent insights and understanding of the neuronal growth cone. The auspicious occasion was funded and hosted by the Juan March Institute, and fittingly held in the country where Cajal first observed specialized formations at the tips of axons and dendrites, and dubbed them “growth cones.” Cajal was equipped with a light microscope, fixed samples of Golgi-stained nervous tissue, and the artistic talent to translate what he saw with the microscope into remarkably accurate drawings. He had the extraordinary prescience to anticipate from static images the range of dynamic functions for which we now have firm evidence. The recent meeting included a vast array of genetic, molecular, biochemical, and imaging techniques, most of which were unheard of in the days of Cajal, all aimed at improving our knowledge of his “battering ram,” which he envisaged also to have “exquisite chemical sensitivity” (Cajal, 1911). As much as scientists prefer to find simple and recurring themes in nature, the overall conclusions of the meeting were that growth cones are extraordinarily complex, that all growth cones are not alike, and that there are multiple pathways for orchestrating essential growth cone behaviors during neural development. Our purpose in this report is to briefly describe the findings presented at the meeting and thereby provide a flavor for contemporary thinking on growth cone biology.

Growth cones are present at the tips of axons and dendrites and at the tips of their developing branches. Axonal growth cones, in particular, are capable of responding to a host of environmental factors that guide

them to their targets (reviewed in Mueller, 1999; Tessier-Lavigne and Goodman, 1996). During development, axons undergo bouts of elongation and retraction, and their growth cones can turn dramatically toward or away from external cues such as chemical factors and guidepost cells. Over the years, scientists interested in growth cones have studied them from several different perspectives. There is great interest in the cytoskeletal machinery inside the growth cone that permits it to move forward, collapse, retract, and change direction. There is also great interest in the environmental factors that initiate such events and the cascade of biochemical events that transduce these cues into cytoskeletal changes. The difficult challenge, as evidenced by the lively discussions at the meeting, is to understand how these biological events are coordinated.

### Cytoskeleton

Discussions on the cytoskeleton seemed to permeate virtually every session of the meeting. There was broad agreement that no matter what signaling cascades were under study, the ultimate effect must be, at least in part, on the actin or microtubule cytoskeletal arrays within the growth cone (for a recent review, see Suter and Forscher, 2000). Actin filaments and microtubules are configured into dense and highly organized arrays that are critical for determining the morphology of the growth cone and for driving its motility. These cytoskeletal arrays are also essential for orchestrating the intracellular transport events that organize the neuronal cytoplasm and for directing the endocytotic and exocytotic traffic. Actin filaments are highly concentrated at the leading edge of the growth cone, forming bundles at the cores of filopodia and in the crosslinked meshwork underlying lamellipodia (Figure 1). Actin filaments undergo assembly and disassembly, and these dynamics have been mentioned for years as the potential target for the biochemical cascades that cause axons to grow, retract, change direction, and branch. It was pointed out in several spirited discussions that both the myosin-based contractility and dynamics of the actin cytoskeleton contribute to changes in growth cone morphology and behavior. Microtubules, which also undergo dynamic assembly and disassembly, form a dense bundle along the length of the axon that extends into the growth cone to “meet” the dense actin cytoskeleton (Figure 1). It has long been recognized that the coordination of the microtubules and actin are at the heart of how growth cone behaviors are orchestrated, but the precise means by which this occurs has been elusive. Indeed, there was broad agreement that ascertaining how these two filament systems engage one another could be the key to understanding the machinery of growth cone navigation.

Reporting on work with vertebrate brain neurons in culture, C. Dotti (Cavaliere Ottolenghi Scientific Institute, Italy) presented studies showing that the actin cytoskeleton of the growth cone is remodeled during axogenesis. Specifically, he showed that the actin cytoskeleton be-

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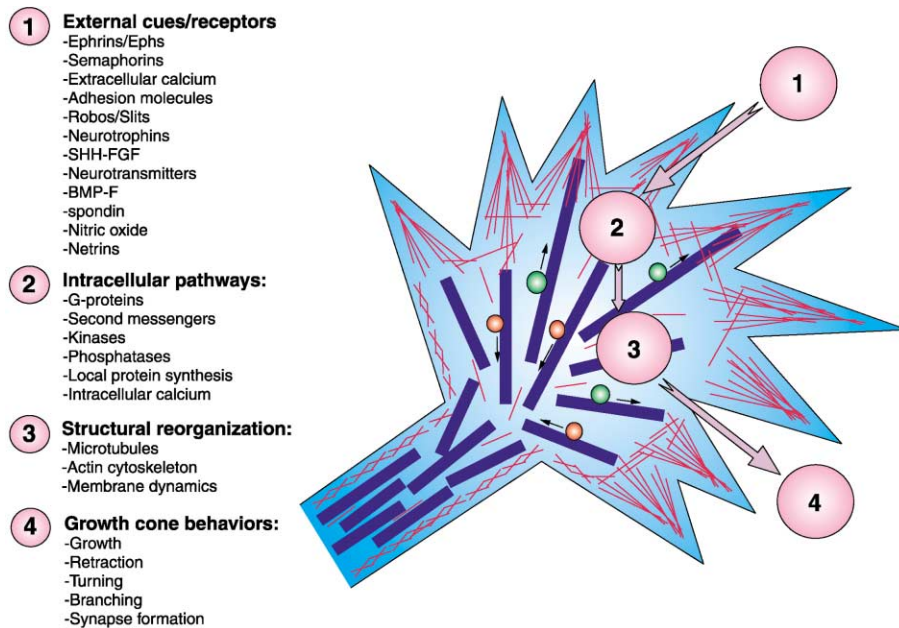


Figure 1. Summary of Signaling Mechanisms at the Growth Cone

External cues result in activation of intracellular signaling pathways, which in turn cause alterations in cytoskeletal structures (actin filaments in red, microtubules in blue) and membrane dynamics. Ultimately, these effects cause changes in growth cone behaviors. Listed here is a sampling of some of the elements of this scenario.

comes more labile to anti-actin drugs and shows diminished phalloidin staining compared to the growth cones of primitive neurites not undergoing axogenesis. These changes in the actin cytoskeleton were posited to permit microtubules to invade different regions of the growth cone, thus accounting for the navigation of the growth cone in a particular direction. In a related presentation, K. Kalil (University of Wisconsin) showed fluorescent microtubules and actin filaments imaged simultaneously in living growth cones undergoing growth and branching. These studies suggested that a subset of the microtubules and actin filaments co-polymerize with one another, providing another means by which the two polymer systems might interact. Two other presentations focused on changes in the microtubule array as growth cones respond to physiological cues. P. Baas (MCP Hahnemann University) showed studies indicating that microtubules do not undergo detectable depolymerization as axons retract in response to nitric oxide, but instead, undergo what appears to be a motor-driven reconfiguration. Indeed, the reconfiguration of microtubules was very similar to that observed in axons induced to retract by manipulation of motor proteins. P. Salinas (Imperial College of Science, UK) showed that microtubules splay apart in addition to showing alterations in stability in response to environmental factors that utilize GSK3 kinase (via the WNT-signaling pathway). These studies demonstrate that alterations in the cytoskeleton that occur within the growth cone are far more complex than can be explained simply by polymerization and depolymerization. There was great interest expressed in the discussions in pursuing both the motor and non-motor microtubule-associated proteins and actin-regulatory proteins that are potential targets for the pathways that induce these changes in the cytoskeleton.

### Extracellular Cues and Their Receptors

Since the discovery of several classes of now “classical” axonal guidance cues in the 90s—such as netrins, semaphorins, ephrins, and slits (Mueller, 1999; Tessier-Lavigne and Goodman, 1996)—it has become clear that these molecules have a broad array of functions (Yu and Bargmann, 2001). In addition, the list of extracellular cues has grown, as evidenced by the array of different ligands and receptors discussed at the meeting. Ephrins and their Eph receptors, known for their role in establishing the anterior-posterior retinotectal topographic map, were discussed extensively. It was previously demonstrated that ephrins, known as repulsive axonal guidance cues, can also act as receptors for “reverse signaling” using the Eph-receptor as the ligand. U. Drescher (King’s College London, UK) presented evidence for ephrin-A’s function in the formation of the vomeronasal (VNO) projection. Ephrin-A5 is more highly expressed on vomeronasal axons from the apical than the basal VNO, and EphA6 is expressed higher in the anterior than the posterior parts of the target. High ephrin-A5-expressing axons project to high EphA6-expressing anterior targets. This indicates that—in this case—ephrin-A5 serves as an attractive axon guidance receptor in the “reverse signaling” mode. Studies using the *in vitro* stripe assay and ephrin-A5 mutant mice support this new role. Interestingly, W. Harris (University of Cambridge, UK) provided new data from C. Holt’s and his laboratory to show that Ephrin B signaling, both forward and reverse, may be important for mapping along the dorsal ventral axis in the *Xenopus* retinotectal system.

Bi-directional signaling was also implicated in the function of receptor tyrosine phosphatase (PTPase) Dlar. Dlar was identified previously as an axonal guidance molecule in the *Drosophila* embryonic nervous sys-

tem. B. Dickson (Research Institute of Molecular Pathology, Austria) presented new data showing that Dlar is essential for *Drosophila* photoreceptor R7 axon projection into a specific layer of the medulla. In *Dlar* mutants, although R7 axons initially project to the correct layer, the axons cannot stabilize their connections and many retract to the R8 layer. Interestingly, single cell mosaic analysis and single cell rescue experiments indicate that Dlar has both a cell-autonomous function in R7 which is dependent on PTPase function, and a cell-nonautonomous function in R8 which is not dependent on PTPase function. Thus, Dlar appears to serve as a receptor as well as a ligand. How does this work? Could Dlar in neighboring cells signal through homophilic interaction? This seems unlikely, as expression of Dlar in R7 alone is largely sufficient for normal targeting even in the absence of Dlar in either R8 or medulla cells. Homophilic interactions may however contribute to LAR function in other systems. E. Macagno (University of California) presented evidence that the leech homolog of Lar (HmLar) plays a critical role in a remarkable parallel growth of multiple processes of comb cells. In normal comb cells, parallel growth cones lead to parallel growth of processes likely via contact-mediated retraction of filopodia from neighboring growth cones. Perturbation of HmLar function via double strand RNA interference (RNAi) results in neighboring processes no longer avoiding each other.

A number of important morphogens that pattern early embryonic development appear to have additional roles in axonal guidance, including household names such as Sonic hedgehog (Shh), bone morphogenic protein (BMP), and WNT proteins. P. Bovolenta (Instituto Cajal, Spain) presented evidence that Shh serves as a repulsive axonal guidance cue for retinal ganglion cell (RGC) axons expressing the Patched receptor. Expression of Shh in specific domains near the midline ensures RGC axons form the optic chiasm at the appropriate location. P. Salinas reported that WNT7a stops cerebellar granule cell axonal growth and stimulates its synaptic maturation. A poster from the laboratory of P. Bovolenta showed that secreted frizzled related proteins (SFRP) stimulate RGC axon growth. SFRP in theory could function by titrating WNT signaling, thus supporting WNT as an axonal stop signal. G. Marques (University of Minnesota) reported the function of a *Drosophila* BMP II receptor in synapse maturation. J. Culotti (Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Canada) provided genetic evidence that *unc-129*, encoding a TGF- $\beta$  family member, is essential for *C. elegans* dorsal circumferential axonal guidance.

It is evident that growth cones listen to different extracellular signals at different stages of their journey, or even at the same time. In fact, *unc-129* was identified in a genetic screen for suppressors of a guidance defect caused by misexpression of the worm netrin receptor Unc-5 (J. Culotti), suggesting that netrin and the TGF- $\beta$ /BMP family of extracellular cues act in a concerted manner. C. Mason (Columbia University) reported the collaborative roles of three different classes of guidance cues/receptors, Slit/Robo, Nr-CAM, and Eph/Ephrins, in ensuring the fidelity of RGC axon guidance at the mouse optic chiasm. Ephrin B2 works to prevent normally uncrossed axons from crossing; Slit expression regulates

where along the midline the crossing axons form the chiasm, whereas NrCAM functions during the crossing step. P. Letourneau (University of Minnesota) presented evidence that one class of extracellular cue could “prime” the response of growth cones to another cue: pretreatment of NGF reduces growth cone collapse in response to subsequent application of Sema 3A. Thus, it will be important not only to explore the signaling mechanisms of individual guidance cues, but also how multiple guidance cues act simultaneously or in sequence to change growth cone behavior. How the actions of multiple guidance cues are integrated and what mechanisms regulate spatially and temporally specific expression of receptors for these cues remain to be explored.

### Intracellular Signaling

How extracellular cues regulate the machinery inside the growth cone to achieve their purpose was a third focus of the meeting, aiming to link ligand-receptor interactions to the cytoskeleton (for a recent review, see Song and Poo, 2001). The Rho family of small GTPases are important intracellular signaling proteins that transduce signals from cell surface receptors to the cytoskeleton. Previous studies using dominant negative mutants have shown that perturbation of Rho GTPases has drastic effects on axonal growth and guidance (reviewed in Mueller, 1999). L. Luo (Stanford University) provided genetic evidence that endogenous Rac proteins are essential for axonal growth, guidance, and branching in *Drosophila* mushroom body neurons. B. Dickson presented data supporting the idea that Racs act downstream of guanine nucleotide exchange factor (GEF) Trio in *Drosophila* photoreceptor axons. Several RhoGEFs—positive regulators of RhoGTPase signaling including *C. elegans* Unc-73, *Drosophila* Trio, and vertebrate Ephexin—have previously been reported to play essential roles for axonal guidance. Rho GTPases are also regulated negatively by Rho GTPase activating proteins (RhoGAPs). L. Luo reported a systematic study of *Drosophila* RhoGAPs using transgenic RNAi and identified p190 RhoGAP as essential for repressing a “retraction signaling pathway” involving RhoA, *Drosophila* Rho-associated kinase (Drok), and phosphorylation of myosin regulatory light chain. Inactivation of p190 RhoGAP results in activation of the RhoA pathway, resulting in axonal retraction.

D. Van Vactor (Harvard Medical School) presented genetic and biochemical evidence that a signaling complex composed of Abl tyrosine kinase, enabled protein, profilin, and the newly identified cyclase-associated protein (CAP) may act downstream of multiple guidance receptors. *cap* exhibits genetic interactions with multiple components in the *slit* repellent pathway at the midline of *Drosophila* embryos. Cap itself is an actin monomer binding protein that antagonizes actin assembly in different cellular contexts. C. Klämbt (University of Münster, Germany) reported that *kette* is essential for *Drosophila* motor axonal guidance. *kette* encodes a multidomain protein that on the one hand is in a complex with Dock/Nck that potentially links to Rho GTPase signaling, and on the other hand binds to several actin binding proteins and actin itself. Elucidating the mecha-

nisms of how the Cap/Ena complex functions and how Kette links different binding proteins together promises to provide additional insights into how signals from cell surface receptors are transduced into cytoskeletal alterations.

Despite the strong focus of the meeting on the cytoskeleton, C. Dotti and M. González-Gaitán (Max-Planck-Institute of Molecular Cell Biology and Genetics, Germany) provided frequent reminders that membrane dynamics are also profoundly important to growth cone behaviors. Dotti noted that, early in axonal development, there is a massive and indiscriminate flow of cytoplasm into the neuronal process that will become the axon, and that this is subsequently replaced by a more selective compartmentalization of membranous components into axons and dendrites. González-Gaitán, focusing on synapse formation and function, discussed the “intermediate endosomal compartment” which acts as a buffer between the membrane released and retrieved during synaptic vesicle traffic. Specifically, he showed that the small GTPase Rab5 determines the kinetics of recycling and the release of the recycled vesicles.

As if to accentuate the vast array of pathways and mechanisms that can affect the growth cone, N. Spitzer (University of California) showed a series of remarkable studies on the effects of calcium transients on growth cone behaviors, something not touched upon in any of the other presentations. These studies showed that rapid calcium transients correspond directly to pauses in growth cone advance, and that such pauses could be experimentally inhibited by locally chelating calcium. There was a great deal of curiosity during the discussion as to how these observations fit with the other messenger systems of the growth cone.

#### Local Protein Synthesis and Metabolism

Perhaps the most unexpected findings reported in the meeting dealt with new roles for mRNA protein synthesis and processing in growth cone guidance. G. Bassell (Albert Einstein College of Medicine) reported a role for the RNA binding protein ZBP1 in the active transport of  $\beta$ -actin mRNA particles into developing axons and growth cones of cultured hippocampal neurons. Perturbation of the transport of this mRNP complex impaired forward motility of growth cones. C. Holt (University of Cambridge, UK) presented striking effects of inhibiting protein synthesis on the *Xenopus* retinal ganglion cell growth cone “turning responses” to multiple attractive and repulsive guidance cues. Evidence for a potential signaling pathway from guidance cues to the phosphorylation of regulators of protein synthesis was presented. These studies suggest that growth cone structure and motility are regulated by mRNA transport and local translation mechanisms. Adding to the role of mRNA transport and protein synthesis in growth cone biology, K. Zinn (Caltech) provided evidence that Pumilio and Nanos play key roles in the development of the *Drosophila* neuromuscular junction. Nanos and Pumilio are well known as regulators of mRNA translation in *Drosophila* early embryo patterning. Both loss-of-function and gain-of-function experiments now implicate these translational regulators in determining the size of synapses. To round out the story, A. Ferrus (Instituto Cajal, Spain)

discussed the importance of protein degradation, and A. Klar (Hebrew University, Israel) discussed the importance of proteolytic processing by serine proteases in growth cone biology. Ferrus showed that *Drosophila ariadne* mutants exhibit defects in axon guidance and synapse maturation. *ariadne* encodes a protein with a RING-finger domain that likely serves as a ubiquitin ligase and physically interacts with a ubiquitin conjugating enzyme. Using nested deletion proteins, Klar showed evidence for the plasmin-mediated release of F-spondin from the extracellular matrix. This effect is crucial for promoting the outgrowth of commissural axons while simultaneously suppressing the growth of motor axons. Future studies of the proteins that must be newly synthesized, degraded, or proteolytically processed for proper growth cone behavior will surely enrich our understanding of the complex biology of neural development. In addition, new axonal guidance genetic screens in zebrafish and worm reported in the meeting (M. Granato, University of Pennsylvania; S. Clark, Skirball Institute) promise to identify new genes important for growth cone signaling.

#### Concluding Remarks

Figure 1 is our attempt to integrate the topics discussed at the meeting into a schematic summary. External cues (most of which involve specific receptors) activate several different but interrelated signaling pathways which give rise to alterations in the cytoskeleton and membrane dynamics. In turn, these alterations act within the growth cone to cause axons to grow, retract, navigate, branch, and form synapses. The vastness of these cues, signals, and pathways is, at the same time, both bewildering and exciting. It is our expectation that the next generation of growth cone biologists, represented by an array of excellent student poster presentations, will continue to piece together the puzzle of the growth cone, the remarkable and yet enigmatic “battering ram” of Cajal.

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