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The Assembly of Neural Circuits

Meeting Report

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The enormous complexity and the incredible precision of neuronal connectivity have fascinated researchers for over a century. This past fall, Lago di Como was the site of a gathering of a diverse group of neuroscientists at the "Assembly of Neural Circuits" meeting, organized by Holly Cline, Barry Dickson, Christine Holt, and Luca Tamagnone and sponsored by EMBO. This meeting highlighted recent findings on the cellular mechanisms of axon guidance, growth cone remodeling, new axon guidance cues, mechanisms of in vivo guidance events, specificity of synaptic connections, and the role of neural activity in circuit development.

1. Cell Biology of Growth Cone Guidance

Progress over recent years has endowed us with the knowledge of a large number of axon guidance molecules and their cognate receptors, and in many cases, the downstream signaling components have also been identified. For the most part, however, the specific cellular mechanisms that mediate guidance remain mysterious.

Endocytosis and Growth Cone Guidance

Several talks focused on the role of endocytosis in growth cone guidance. For instance, the ephrins are membrane bound guidance molecules that are well known for their potent repulsive functions and come in two classes: the A class, which are tethered to the membrane through glycosylphosphatidylinositol (GPI) linkage and signal through EphA receptors; and the B class, which are transmembrane proteins and signal through EphB receptors. EphA4 is an unusual EphA that also mediates B class ephrin signaling. An intriguing question is how these ligand-receptor systems lead to repulsion as these membrane bound molecules bind tightly with each other and initially bring together the growth cones and their targets. For the A subgroup of ephrins, it was proposed that the metalloprotease Kuzbanian cleaves these GPI-linked ephrin-As and thus releases them from the cell surface (Hattori et al., 2000). However, protease cleavage of the transmembrane B class ephrins was found to be very inefficient. Rüdiger Klein of the Max-Planck Institute of Neurobiology, Munich, Germany, reported compelling evidence that bidirectional transendocytosis occurs between cells and neurons expressing ephrin-B and EphB upon cell-cell contact (Zimmer et al., 2003). This endocytic process requires the intracellular domains of ephrin-B and EphB for both the reverse and forward directions, respectively. Interestingly, ephrin-As can also trigger endocytosis when engaged by EphA4, suggesting that proteolysis and endocytosis may function in parallel. These studies open up an interesting question of whether endocytosis is simply involved in detachment or may instead participate actively as part of a signaling mechanism. Endocytosis may not be a mechanism exclusive for the ephrins. In fact, endocytosis has been shown to occur during Sema3A-mediated growth cone collapse (Fournier et al., 2000; Jurney et al., 2002). Christine Holt of Cambridge University presented data to show that reverse-direction endocytosis occurs in retinal growth cones when EphB2-Fc is presented to ephrin-B1-expressing retinal cells and that endocytosis is functionally required for growth cone collapse. This reverse endocytosis is a rapid process that is only triggered by unclustered EphB2 ectodomains and requires proteosome function (Mann et al., 2003). Interestingly, forward signaling that leads to growth cone collapse appears to require clustered ephrin-B1 ectodomain and is proteosome independent. Endocytosis was not detected in the forward direction. These results illustrate interesting differences in ephrin/Eph signaling systems in two directions. Although the functional consequences of these distinctions are not yet clear, these differences may provide an opportunity to allow regulatory signals to exert specific or different influences on the two partners, leading to different downstream events in the two cells that are in contact.

Membrane Compartmentalization

In addition to being expressed in the surrounding environment, guidance cues are also frequently found expressed on the navigating axons themselves and often are together with their receptors, an observation that seems enigmatic. Although there has been much speculation, in most cases, the functional role of this axonal expression has not been resolved. However, in the case of at least one family of guidance cues, the ephrin/Eph receptors, there is emerging evidence for a role for the Eph receptor ligand ephrin-A in the regulation of growth cone responsiveness. It was observed some time ago that nasal retinal ganglion cell (RGC) growth cones gain responsiveness to posterior tectum when ephrin-As are removed from these axons, while temporal retinal axons lose their sensitivity when ephrin-As are expressed on them (Hornberger et al., 1999; Feldheim et al., 2000). Uwe Drescher of the MRC Center for Developmental Neurobiology, King's College London, presented evidence to suggest that the role of ephrin-A in RGC axon growth cones may be to keep the EphA receptor function at bay by regulating the trafficking of EphA receptors to lipid raft domains. Lipid rafts are microdomains in the plasma membrane enriched in cholesterol and sphingo-

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lipids, where a subpopulation of membrane proteins, including the GPI-anchored proteins, tend to reside (Munro, 2003). Drescher provided data that GPI-linked ephrin-A is able to bring EphA receptors into a particular type of lipid raft and keep the repulsive signaling inhibited. This recruitment appears to be due to a *cis* interaction between ephrin-A and EphA receptor independent of the N-terminal ligand binding domain on the EphA receptor.

Cytoskeletal Changes

The localized reorganization of the actin cytoskeleton plays a central role in growth cone motility, and there is much evidence to suggest that the Rho family of small GTPases and their regulators guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) play key roles in this process. Although many such GTPases and their regulators have been identified and implicated in guidance in various contexts, precisely how they "talk" to the actin cytoskeleton is not clear. One candidate output signal is the actin depolymerizing factor (ADF)/cofilin (Sarmiere and Bamburg, 2004). ADF/cofilin is inactivated by kinases such as LIM-kinase 1 (LIMK1), downstream effectors of the Rho GTPases, and is reactivated by a family phosphatases, Slingshot, that also bind to F-actin (Niwa et al., 2002). Liqun Luo (Stanford University) provided the first compelling in vivo evidence of the role of *Drosophila* Slingshot in mushroom body axon growth and morphogenesis. While this work provides in vivo evidence that actin depolymerization is a crucial regulatory step in growth cone motility, the studies also emphasized that multiple signaling pathways are required to regulate the cytoskeleton downstream of Rho GTPases.

2. The Dynamic Growth Cone

A fundamental feature of the growth cone is that its sensitivity to environmental cues undergoes dynamic changes during in vivo pathfinding. For example, the midline is a well-established intermediate target where changes of growth cone responsiveness to specific guidance cues have been documented (Dickson, 2002). Several mechanisms have been proposed that may explain the changes of responsiveness to guidance information at midline intermediate targets, such as silencing of DCC signaling by Robo (Stein and Tessier-Lavigne, 2001) to turn off Netrin-1 responsiveness, sorting of Robo by Comm to turn off Slit responsiveness (Keleman et al., 2002), and regulation of new protein synthesis at the midline (Brittis et al., 2002). The dynamic nature of the growth cone is likely to reflect a more general intrinsic property. In fact, it is possible that the growth cone itself might undergo rhythmic remodeling while climbing up a simple chemoattractant gradient, a phenomenon coined "growth cone adaptation" (Ming et al., 2002). It has been proposed that growth cones are constantly being desensitized to guidance cues and resensitized again. Ming et al. (2002) have shown that resensitization is dependent on local protein synthesis, and in the context of an in vitro turning assay of embryonic *Xenopus* spinal neuron axons in response to a Netrin-1 gradient, this results in a "zig-zag" trajectory. William Harris of Cambridge University reported that desensitization and resensitization also occur in *Xenopus* retinal axons, and in fact, in this system, the time course of desensitization and resensitization of retinal axons occurs at a much faster rate than has been observed for embryonic spinal axons.

3. How Many Cues to Wire the Nervous System?

The enormous complexity of neural circuits raises a question of how many molecular guidance cues or recognition molecules are required to wire our dauntingly complex brain? Four types of molecules have been shown to guide axons: diffusible attractants, diffusible repellents, contact attractants, and contact repellents (Tessier-Lavigne and Goodman, 1996). Bidirectional signaling in growth cone guidance, changes of responsiveness along a trajectory, and graded distribution of guidance cues can all diversify or maximize the function of axon guidance molecules in wiring the nervous system. Some axon guidance receptors, such as the *Drosophila* immunoglobulin superfamily member Dscam, have an enormous number of splice variants (Schmucker et al., 2000). Dscam has been shown to play multiple roles in axon pathfinding, morphogenesis, and target selection. In this meeting, Larry Zipursky from the University of California, Los Angeles reported a novel function of Dscam in axon-axon interaction during the development of mushroom body axon development. Younger neurons project axons in the center of the peduncle, a thick nerve in the mushroom body transiently expressing Dscam, which mediates homophilic attractions and is essential to the organized projection of older versus younger neurons. Axon-axon interaction or selective fasciculation is an important mechanism in nervous system wiring and yet very little is known about it. Interestingly, Cori Bargmann from the University of California, San Francisco reported that Syg-1 and Syg-2, also members of the immunoglobulin superfamily that are expressed in HSNL neuron and vulval epithelium, respectively, mediate specific axon target selection of HSNL neurons to their vulval muscles in determining the location of specific synapses (Shen and Bargmann, 2003).

A recent theme in axon guidance has been that several classes of signaling molecules that were previously shown to play important roles in earlier embryonic development and patterning also participate in axon guidance. Several recent studies have shown that the classic morphogens, such as the bone morphogenetic proteins (BMPs) (Augsburger et al., 1999), Sonic Hedgehog (Shh) (Charron et al., 2003; Osterfield et al., 2003), and Wnts (Yoshikawa et al., 2003), also act as guidance cues later in development. In this meeting, Yimin Zou of the University of Chicago reported that the spinal cord commissural axons initially remain unresponsive to Wnts as they project along the dorsal-ventral axis before midline crossing but become attracted by Wnts immediately after midline crossing to project anteriorly to the brain (Lyuksyutova, 2003). Paola Bovolenta of the Instituto Cajal (Madrid Spain) also provided evidence that SHH can act as a guidance factor for retinal ganglion cell axons. Shh is expressed at the borders of the optic chiasm and defines a constricted pathway for RGC axons within the ventral midline, possibly in collaboration with the extracellular matrix molecule Vitronectin (Trousse et al., 2001).

4. Wiring Up the Nervous System

The optic chiasm has long been a focus of attention in studying the midline crossing decisions in the establishment of binocular vision. Previous studies from Christine Holt's lab suggested that ephrin-B present in the optic chiasm of the metamorphosing tadpole plays a role in sorting the ipsilateral and contralateral axons (Nakagawa et al., 2000). Carol Mason of Columbia University reported that the ephrin-B2-EphB1 system is also a major player in the divergence in mammalian optic chiasm (Williams et al., 2003). Ipsilateral axons are greatly reduced in the absence of EphB signaling. Interestingly, some ipsilateral axons still exist even in EphB1/EphB2/EphB3 triple knockout mice, suggesting that perhaps cues other than ephrin-B2 also exist to control the midline crossing of RGC axons. A transcription factor, Zic2, is necessary and sufficient for the regulation of ipsilateral RGC axon repulsion by the optic chiasm (Herrera et al., 2003). Indeed, transcriptional regulation of neuronal connections can be very elaborate. Silvia Arber of Biozentrum and the Friedrich Miescher Institute, Basel, Switzerland, showed that altering the normal late function of ETS transcription factors such as Pea3 by expressing a potent transcriptional activating variant of Pea3 during early development resulted in abnormal projections of dorsal root ganglion axons in the spinal cord. The same Pea3 variant can rescue the abnormal projection of proprioceptive afferents in Er81 mutants at a later stage. These results suggest that temporal precision in transcriptional regulation is likely an integral part of the regulatory mechanisms in the development of neuronal connectivity. Michael Bate of Cambridge University presented his studies stressing the function of receptor codes in addition to transcription factor codes in the formation of complex arborization patterns (Zlatic et al., 2003).

5. Specificity of Synaptic Connections

The development of neural circuits hinges not only on cell fate determination and pathfinding but also depends crucially on synapse formation. After axons reach their final destination, they eventually form specific connections with only a subset of target cells in the immediate vicinity. Cori Bargmann and colleagues at UCSF examined the cell interactions that mediate the formation of a stereotyped set of synapses in *C. elegans* (Shen and Bargmann, 2003). They found that interactions between the pre- and postsynaptic cells are not sufficient for the development of these connections. Instead, an epithelial "guidepost cell" signals through a membrane bound ligand for a transmembrane receptor on the presynaptic neuron to direct synaptic vesicle clustering at a particular site. The presynaptic cell then triggers the recruitment of its postsynaptic partner. This work gives a different spin to the question of specificity of synapse formation, since nonneuronal cells seem to be entering the fray in determining where neuronal connections are made.

A similar question was addressed by Herwig Baier's lab (also at UCSF) in the zebrafish inner plexiform layer (IPL), where the circuitry that produces a distinct ganglion cell response (ON or OFF response) is spatially segregated into inner and outer sublaminae. They found that GFP-labeled amacrine cells can usually project to the correct target region even in the absence of their major postsynaptic targets (RGCs), which are entirely missing in the mutants under study (lakritz) (Kay et al., 2001). Nonetheless, sublamination of the IPL was severely disrupted, indicating that postsynaptic signals do play some role in establishing the circuit. Large-scale genetic screens for mutations affecting IPL organizations are underway, and first results have begun to reveal the cellular and molecular mechanisms that underlie synaptic target choice in the retina.

Synapse specificity is determined not only by which pair of neurons form synaptic partners but also by where the synapses are formed. Subcellular domain-specific synapse is a salient feature of innervations made by GABAergic neurons (Somogyi et al., 1998). For example, in cerebellum, basket interneurons make synapses precisely at the axon initiation segment (AIS) of Purkinje cells. Josh Huang and colleagues at the Cold Spring Harbor Laboratory identified a gradient of an immunoglobulin cell adhesion molecule along the AIS-soma-dendritic axis of the Purkinje cell as a potential cellular mechanism for achieving such specificity. In mice deficient in ankyrinG, a membrane protein that is itself exclusively localized at AIS, the gradient was abolished and the formation of AIS-specific synapses was impaired. In addition, ectopic expression of this cell adhesion molecule along Purkinje axons correlated with the mistargeting of basket cell terminals. This implicates that an ankyrin-based gradient of cell adhesion molecules may direct the specific innervation at Purkinje cell AIS by basket interneurons.

6. Activity-Dependent Development of Circuits

The formation of precise neural circuits not only depends on the molecular cues but also relies on normal neuronal activity in many parts of the nervous system. It has been proposed that correlated activities of the pre- and postsynaptic neurons lead to the stabilization of the connection formed between them. Using time-lapse imaging of RGC axons in *Xenopus* tadpoles, Hollis Cline at the Cold Spring Harbor Laboratory directly examined how correlated neural activity governs structural modulation in vivo (Ruthazer et al., 2003). After unilateral ablation of the optic tectum, ipsilateral retinal axons were forced to innervate the contralateral tectum and compete with inputs from the contralateral eye. During this competition, which will eventually lead to the segregation of afferents into eye-specific bands, they found that axons from both eyes added branch tips with nearly equal probability but eliminated them preferentially from territory dominated by the opposite eye. This selective branch elimination was abolished by NMDA receptor blockade. These results suggest a correlation-based mechanism by which visual experience directly governs axon branch dynamics through selective stabilization of synapses where the activity pattern of pre- and postsynaptic cells is correlated.

Carla Shatz and colleagues at the Harvard Medical School also looked at activity-dependent rearrangement of synaptic connections, but in cat visual cortex. They focused on the subplate, which forms a transient circuit required for the development of axonal projections be-

tween thalamus and cerebral cortex, since early subplate ablation prevents the innervation of thalamic axons into layer 4 (Ghosh et al., 1990), whereas later ablation prevents the segregation of thalamic axons into ocular dominance columns (Ghosh and Shatz, 1992). They found that ablation of subplate neurons after the arrival of thalamic inputs led to profound functional deficits in the visual cortex (Kanold et al., 2003). Functional orientation maps were disrupted or absent, and neurons exhibited much weaker orientation selectivity. Additional in vitro slice recordings showed that the ablations resulted in reduced efficacy of thalamocortical synaptic transmission, which is consistent with the lower expression of GluR1 in layer 4 of the ablated area. Why are subplate neurons necessary for the functional synaptic maturation and remodeling of cortical neurons? The current model proposes that subplate neurons provide excitatory inputs to cortical neurons, the activation of which may be necessary for the progressive strengthening of synapses made by thalamic afferents.

Neuronal activity influences many aspects of nervous system development through Ca^{2+}-dependent signaling. For some long-term effects, such as activity-dependent dendritic growth, long-term plasticity of the sensory system, and memory consolidation, Ca^{2+}-dependent transcription is involved. Using a strategy of transactivation trap, Ghosh and colleagues, then at Johns Hopkins University, screened for Ca^{2+}-activated transcription factors (Aizawa et al., 2004). One of the novel factors that was cloned, calcium-responsive transactivator (CREST), is expressed in the developing cortex, hippocampus, and cerebellum. It binds to both CBP and BAF250, suggesting that CREST may regulate transcriptional activation via Ca^{2+}-dependent chromatin remodeling. CREST-deficient mice have a smaller cerebral cortex and cerebellum, aberrant hippocampal organization, and retarded dendritic growth of cortical and hippocampal neurons. CREST mutant cells are also compromised in Ca^{2+}-dependent dendritic growth in vitro. These results indicate that CREST plays a critical role in cortical neuron development. Identifying Ca^{2+}-activated transcription factors will greatly enhance our understanding of the molecular mechanisms for the activity-dependent development.

Along another line of mechanistic study, Erin Schuman looked more closely at the role of dendritic protein synthesis in long-term synaptic plasticity. Work in her lab at the California Institute of Technology has demonstrated that isolated dendrites of mature hippocampal neurons can still synthesize proteins (Aakalu et al., 2001). Using GFP-based protein synthesis reporter, they were able to demonstrate that electrical activity or application of neurotransmitters and neuromodulators can influence protein synthesis. Currently, the utility of synthesis reporters based on puromycin, an amino-acyl tRNA analog, is being investigated. Those novel reporters can overcome the problem of the rather slow maturation time of GFP, which limits its use as a reporter of rapid events. These novel techniques may prove useful in dissecting the spatial and temporal characteristics of activity-induced changes in synaptic efficacy.

Concluding Remarks

This meeting reflected recent exciting progress in understanding circuit development and also predicted new frontiers for the field. In closing, we highlight just a few of the problems that are being tackled. As more and more molecular guidance cues are identified, the mechanisms of how these guidance signals are integrated to influence axon behavior and pathfinding will become a major focus. From a cell biological perspective, it still remains quite mysterious how these different molecular signaling pathways operate in the cellular context and how these signals are translated to the mechanical changes required for growth cone remodeling and navigation. Future efforts will also be needed to decipher how growth cones are able to respond to small concentration changes in a molecular gradient. Once axons reach their targets, the mechanisms determining the specificity of synaptic connections are still quite unknown. Are they regulated by similar molecular cues that guide axons or by a separate class of molecules? It is clear that neural activity plays an important role in circuit development, but much less is known about how activity shapes circuits at the molecular and cellular level. These questions will continue to be addressed in this fast-growing field of investigation in the coming years, and we look forward to meeting again at the Cold Spring Harbor "Axon Guidance and Neural Plasticity" meeting in the fall.

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