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Making Connections in the Fly Visual System

Review

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Understanding the molecular mechanisms that regulate formation of precise patterns of neuronal connections within the central nervous system remains a challenging problem in neurobiology. Genetic studies in worms and flies and molecular studies in vertebrate systems have led to an increasingly sophisticated understanding of how growth cones navigate toward their targets and form topographic maps. Considerably less is known about how growth cones recognize their cellular targets and form synapses with them. Here, we review connection formation in the fly visual system, the methodological approaches used to study it, and recent progress in uncovering the molecular basis of connection specificity.

> The complexity of the insect retina is something stupendous, disconcerting and without precedent in other animals…Compared with the retina of these apparently humble representatives of life…the retina of the bird, or the higher mammal appears as something coarse, rude, and deplorably elementary.
>
> —Ramon y Cajal (1937), Recollections of My Life

Flies are highly visual organisms that can detect color, motion, and polarized light, as well as geometric patterns. As Cajal discovered nearly a century ago, the deceptively simple reiterated pattern of cells in the fly eye conceals a pattern of neuronal connections of staggering complexity (Cajal, 1915). This structure underlies the sophisticated processing capabilities of the fly visual system. The detection and processing of visual stimuli results from the computational functions of neurons within the optic ganglia and the complex patterns of interconnections between them (see Giurfa and Menzel, 1997).

These patterns of connections, like complex patterns of neuronal connections more generally, can be thought of as emerging in three distinct steps: guidance to the target field, choice of the appropriate target from within the local environment, followed by assembly of a functional synapse. Studies in vertebrates and invertebrates support the notion that growth cones navigate toward their targets by detecting and integrating multiple signals; indeed, they appear to execute a series of simple choices defining their trajectory toward their targets (reviewed in Tessier-Lavigne and Goodman, 1996; Flanagan and Vanderhaeghen, 1998; Yu and Bargmann, 2001). In addition, studies on the vertebrate neuromuscular junction, the behavior of cortical neurons in culture, and genetic studies in both worms and flies have begun to define at a molecular level the pathways involved in directing the localization and activation of proteins involved in neurotransmitter release and response (reviewed in Jin, 2002; Murai and Pasquale, 2002; Sanes and Lichtman, 2001).

By comparison with guidance to the target and synaptogenesis, relatively little is known about how growth cones, once within the target field, select specific targets with which to form synaptic connections. It is generally believed that initial patterns of connectivity are crude and that they are then refined by neural activity (Katz and Shatz, 1996). It is clear, however, that extraordinary precision and fidelity can be achieved through genetically hard-wired mechanisms (Trujillo-Cenoz and Melamed, 1966; Braitenberg, 1967; Lin et al., 2000). It is our view that in many systems precise interactions between cell surface determinants are genetically programmed to generate the appropriate pattern of connections.

The question of how many different cell surface recognition molecules are needed to determine the pattern of connections between neurons has been a long-standing issue in the field. In principle, two different mechanisms can be envisioned. A small number of recognition molecules may be utilized in a dynamic fashion. That is, a core recognition system may exist that underlies the connections of many different types of neurons, and the precise spatial and temporal control of its activity or quantitative differences may specify connectivity patterns. This type of mechanism may underlie the formation of topographic maps in the vertebrate visual system. Here, the point-to-point mapping of millions of identical neurons differing only in their position onto an array of functionally equivalent targets is achieved using gradients of cell surface molecules on both afferents (i.e., Eph A receptors) and target neurons (Ephrin A ligands). Alternatively, connection specificity may be determined by a large set of unique cell surface recognition molecules or molecular labels that act in a lock-and-key type fashion. The targeting of vertebrate olfactory receptor neurons to specific glomeruli may be dependent upon this type of mechanism, as instructive cues are provided by a diverse family of seven-transmembrane domain proteins, the odorant receptors (Mombaerts et al., 1996; Wang et al., 1998). These observations raise the possibility that very different strategies regulate the recognition that underlies connection specificity.

To gain insights into the molecular mechanisms regulating connection specificity, several laboratories have taken genetic approaches to dissecting this process in the *Drosophila* visual system. The patterns of neural connections in the fly visual system are precise, com-

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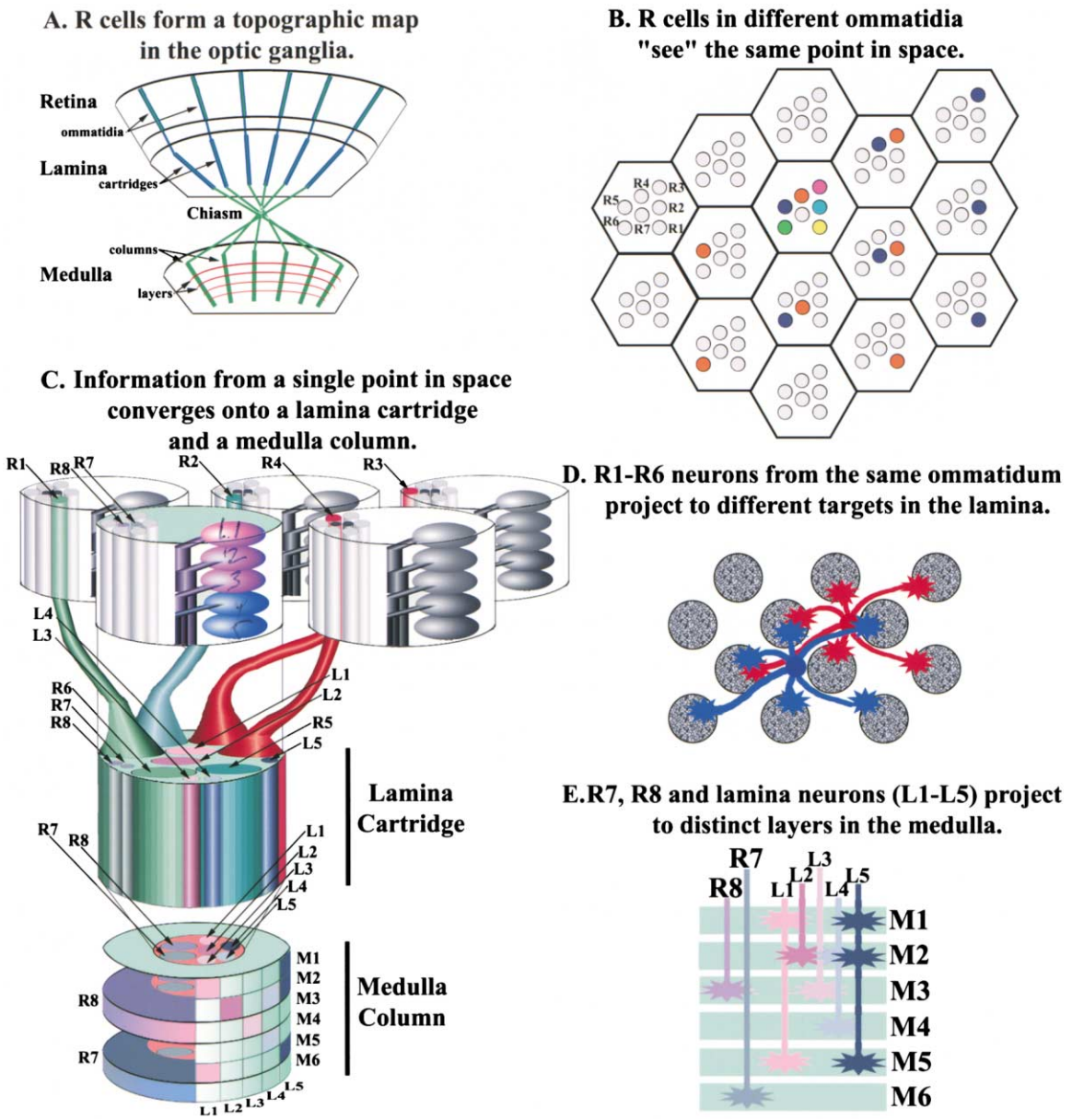

Figure 1. Connection Specificity in the Fly Visual System

(A) R cells elaborate a topographic map, and different R cells terminate in one of two different optic ganglia, the lamina or medulla. This panel shows a horizontal view of the adult retina and the lamina and medulla. Each eye contains about 800 simple eyes or ommatidia (light blue) containing eight photoreceptor neurons (R cells). R1-R6 cells innervate the first optic ganglion, the lamina, to form an array of synaptic units, called cartridges (dark blue). R7 and R8 axons innervate the second optic ganglion, the medulla, forming layered columns (green).

(B) Six different R cells in six different ommatidia "see" the same point in space. Two such groups (marked in orange and blue) are indicated. This panel shows a schematic representation of a section taken tangential to the surface of the adult retina. Each ommatidium appears as a hexagon. R1-R6 and R7 are visible within this plane of section. R8 lies directly beneath R7. R1-R6 cells and the R7/R8 pair within a single ommatidium each look at a different point in space, represented by different colors, and connect to distinct targets in the brain. R cells that look at the same point in space are distributed over the retinal surface and converge on common targets (see panel [C]).

(C) Schematic structure of axon bundles within individual units in the lamina and medulla. All of the R cells and lamina interneurons that see (R cells) or respond to signals (lamina neurons) from the same point in space are shown in color. Uppermost structures represent ommatidial bundles associated with columns of five lamina interneurons. The anatomy of a single lamina cartridge is shown in the middle schematic, while the anatomy of a single medulla column is displayed in the lowest part of the diagram. The R1-R6 neurons form en passent synapses with processes from lamina neurons. Within a medulla column, different R cells and lamina interneurons terminate at distinct layers.

(D) The R1-R6 connection pattern in the lamina as viewed from two bundles of axons (indicated in red and blue) from two neighboring ommatidia. Each R cell from a given ommatidium innervates a single, distinct target (gray circles) located in an invariant position relative to the ommatidial bundle. By precisely overlapping two identical patterns of projections made by R cells from neighboring ommatidia, R cells that look at the same point in space converge on a common target.

(E) R7 and R8 axons, as well as the axons of lamina interneurons, innervate distinct layers in the medulla. The precise positions of axons within the medulla column are currently unknown. Each column also receives axons and dendrites from different classes of medulla neurons (not shown).

plex, and have been described at the level of individual identified neurons (Meinertzhagen and Hanson, 1993). Genetic tools available in *Drosophila* and, in particular, tailored for study in the fly visual system provide powerful approaches to the molecular dissection of this process (Wolff et al., 1997; Newsome et al., 2000a; Lee et al., 2001).

R Cells Form Precise Patterns of Connections

The fly visual system comprises the compound eye and the optic ganglia, the lamina, medulla, and the lobula complex (Figure 1A). Each region contains a wealth of different neuronal cell types with distinct morphologies and patterns of connectivity (Meinertzhagen and Hanson, 1993). The compound eye comprises an array of some 800 simple eyes or ommatidia, each containing eight R cells arranged in a stereotyped fashion (Figure 1B). These cells fall into three classes. R1-R6 cells express a rhodopsin with a broad absorption in the green range, while R7 and R8 neurons express rhodopsins that absorb in the ultraviolet and blue range, respectively. R cells innervate distinct layers in the optic ganglia. R1-R6 neurons form synaptic connections in the first optic ganglion, the lamina, while R7 and R8 axons project through the lamina and terminate in two separate layers in the medulla (Figures 1A and 1C).

The extraordinary precision of neuronal wiring in the fly visual system ensures that each synaptic unit in the lamina and in the medulla represents a single point in space arranged in a smooth topographic map (Figures 1A and 1C). A complex pattern of R cell connections is required to achieve this simple mapping function. Due to the curvature of the eye and the precise geometric arrangement of R cells within an ommatidium, six different R1-R6 cells in six different ommatidia (i.e., an R1 in one ommatidium, an R2 in another, etc.) "see" the same point in space (Figure 1B). In addition, an R7 and R8 pair from yet a different ommatidium also see this point in space. Through a precise interweaving pattern of fibers between the retina and the lamina, the six R1-R6 cells that see the same point in space converge onto a single group of lamina neurons and synapse with them (Figures 1C and 1D; Vigier, 1909; Trujillo-Cenoz, 1965; Trujillo-Cenoz and Melamed, 1966; Braitenberg, 1967; Kirshfeld, 1967; Meinertzhagen and Hanson, 1993). Synaptic units within the lamina are referred to as lamina cartridges. Lamina monopolar neurons (L1-L5) from a single cartridge project, in turn, to distinct layers within a radially oriented synaptic unit in the medulla, called a column (Figures 1C and 1E). Each column also contains the synapses of the R7 and R8 neurons that see the same point in space. In this way, each medulla column receives input from a single point in space, directly from R7 and R8 and indirectly from R1-R6 via lamina neurons.

During development of both the lamina and medulla, R cell growth cones are confronted with a multitude of axons and dendrites from which they reproducibly select specific targets (Meinertzhagen and Hanson, 1993). Within the lamina, for example, each R1-R6 growth cone confronts many R cell and lamina cell surfaces (Meinertzhagen and Hanson, 1993). These include different lamina monopolar and amacrine neurons, lamina glia, tangential neuronal fibers originating in the medulla cortex, centripetal fibers from regions between the medulla and deeper regions of the visual system, and wide-field serotonergic neurons originating in the central brain. Similarly, each medulla column contains processes from an even more diverse collection of neurons (Meinertzhagen and Hanson, 1993). Since R cell axons make synaptic connections with only a small subset of the neuronal cell surfaces that they encounter, robust mechanisms that enable neurons to distinguish between appropriate and inappropriate partners are required.

This exquisite specificity is achieved independent of visual input (Barth et al., 1997; T.R.C. and S.L.Z., unpublished data). It is not known whether spontaneous electrical activity, as in the connections between retinal ganglion cells and their targets in the lateral geniculate nucleus in the vertebrate visual system (Sretavan et al., 1988), contributes to R cell connection specificity. Nevertheless, these findings suggest that intrinsic cellular recognition mechanisms underlie the precision and fidelity of these connectivity patterns.

The precision of neuronal connectivity suggests a complex cellular recognition process that permits specific neurons to distinguish between appropriate and inappropriate cell surfaces. Genetic and developmental studies that we review here have provided cellular and molecular insights into how this specificity is achieved. First, the step-wise assembly of the visual system reduces the demands for molecular specificity by presenting R cell growth cones, at least in some contexts, with only a rather limited number of alternative targets. Second, surprisingly, interactions between afferents play a key role in target specificity for at least one class of visual system neurons. Third, an N-cadherin-based cell adhesion system plays a central role in regulating connection specificity at discrete steps. We propose that this represents an evolutionarily conserved mechanism contributing to target recognition. And finally, recent studies beginning with molecular and genetic analyses in the fly visual system have uncovered a family of guidance receptors in *Drosophila* that exhibit extraordinary molecular diversity generated by alternative splicing. This has led to the proposal that this diversity contributes to the exquisite specificity in connectivity in the fly central nervous system.

R Cell Growth Cones Induce Target Development

The establishment of the R cell projection pattern reflects a complex dialog between R cell axons and neurons and glia within the target field (see Salecker et al., 1998). In broad terms, R cell axons provide cues that induce the development and differentiation of the target cells. These, in turn, provide guidance and targeting signals to R cell axons that direct R cell growth cones toward their postsynaptic targets. This interplay provides mechanisms for coordinating the development of pre- and postsynaptic cells.

The establishment of a topographic map in which R cells in adjacent ommatidia project to adjacent targets largely reflects the spatiotemporal order of ommatidial assembly. R cell differentiation in the eye disc occurs in a sequential fashion, with posterior ommatidia differ-

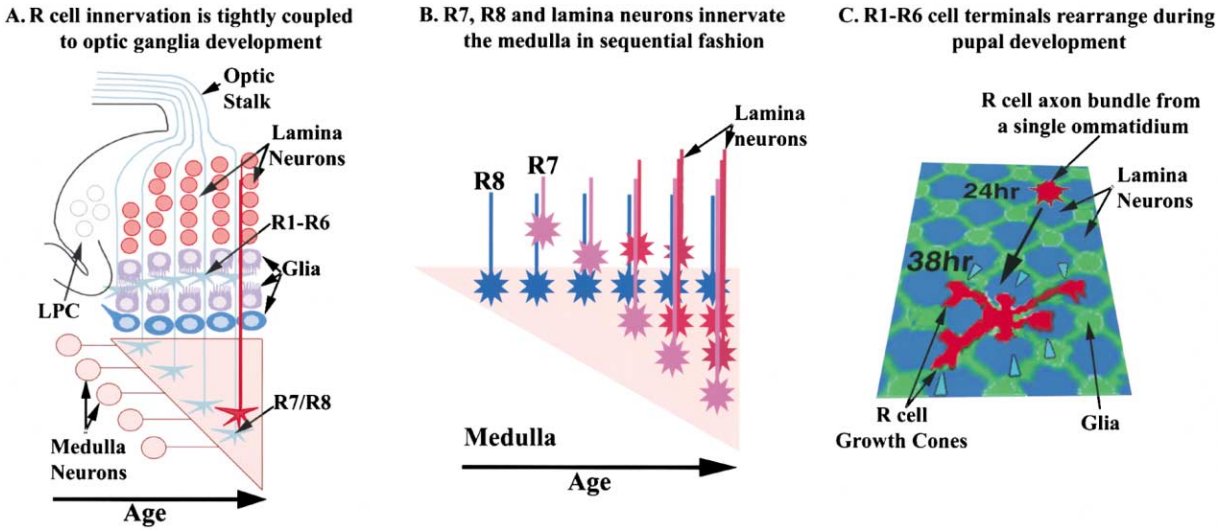

Figure 2. The Development of R Cell Connections in the Fly Visual System

(A) R cells innervate the lamina and medulla in a sequential fashion. The retina (not shown) develops as a wave sweeping across the retinal primordium. As a consequence, R cells project into the optic stalk and innervate the lamina and medulla in sequential fashion. R cell axons in the left part of the lamina are the youngest, with a gradient of increasing developmental age from left to right. Similarly, R7/R8 axons in the medulla are of different ages. Oldest axons are at the bottom of the medulla. As R cells enter the developing lamina, they come into close contact with the lamina precursor cells (LPC). Signals from R cell axons induce lamina neuronal development, thereby precisely matching the number of R cell axon bundles and lamina targets (see text).

(B) R7, R8, and lamina monopolar neurons project in a specific order into developing medulla columns. R8 axons project from the eye through the lamina into a superficial layer within the medulla. R7 growth cones extend along the surface of R8 axons from the same ommatidium and terminate just past the R8 growth cone layer. As lamina neurons begin to differentiate, they too send axons within the R8/R7 bundle and terminate in layers between the R7 and R8 layers. This intercalation accounts in part for the increased separation of R7 and R8 growth cones seen in the adult. Initially, the R7 and R8 growth cone terminals are separated by 2–3 μm; in the adult, these terminals are separated by about 10 μm.

(C) R1-R6 axons (red) project to lamina targets in two steps. Initially, each cluster of R cells in the retina sends a single bundle of axons to the lamina. Lamina glial cells (green) act as intermediate targets for R1-R6 growth cones. The growth cones of the R1-R6 axons terminate as a single cluster. These clusters remain until some 24 hr of pupal development. Between 24 and 38 hr, growth cones extend over the surface of the lamina to their appropriate targets. They then project down into the developing cartridge, assuming their adult trajectories (see Figure 1C).

entiating first, followed by more anterior ones. As R cells differentiate, they extend axons posteriorly through the optic stalk and into the developing optic ganglia (Figure 2A). The orderly ingrowth of R cell axons is mirrored by the accretion of neuronal and glial elements in the target. The number of ommatidia is matched to the number of postsynaptic units in the lamina and medulla. Matching reflects the intimate relationship between R cell and target development. R cell axons produce Hedgehog (Hh) protein, which induces lamina neuronal precursor cell division (Huang and Kunes, 1996), and Spitz, an EGF-like ligand, which induces neuronal differentiation (Huang et al., 1998). R cell axons are also required for lamina glial cell development through an as yet unknown signal (Perez and Steller, 1996). Hence, R cell afferents control the numerical matching of pre- and postsynaptic elements by providing signals that induce the proliferation of neuronal precursors and trigger their differentiation.

R cell axon outgrowth follows the order of R cell differentiation within the ommatidium. R cell axons from the same ommatidium form a single fascicle (Wolff et al., 1997). The R8 growth cone extends first, followed by R1-R6 and then R7. The initial posterior extension of R8 from the eye disc is dependent upon interactions with a subclass of retinal glial cells (Hummel et al., 2002). R8 growth cones extend through the optic stalk and into the lamina prior to the appearance of differentiated lamina neurons and glia. As R8 cells express Hh, it is likely that they induce lamina neuronal precursor cell division. After R8 axons pass through this region, they terminate on axons from differentiating medulla interneurons. R1-R6 axons project along the surface of the R8 axon, through the lamina neuronal precursor cell layer, where they terminate between two layers of lamina glial cells (Perez and Steller, 1996). R7 axons then extend along the bundle through the lamina and into the medulla where they terminate 2–3 μm beyond the R8 terminals (Clandinin et al., 2001) (Figure 2B). During subsequent development, R8 and R7 terminals become separated by about 10 μm, largely through the intercalation of growth cones and dendrites of optic ganglion neurons (C.-H. Lee and S.L.Z., unpublished data). R1-R6 growth cones project to their lamina target neurons during pupal development (Figure 2C) (Meinertzhagen and Hanson, 1993). R cell transformation experiments change the targeting of these neurons without affecting the timing of axon outgrowth. For instance, an R7 neuron transformed into an R1-R6 neuron still extends its axon into the target at the time appropriate for an R7 neuron, but its axon termi-

nates in the lamina as would an R1-R6 cell (Basler et al., 1990). Thus, the layer-specific targeting of different R cell subclasses does not simply reflect the order of R cell innervation.

Glial Cells Are Intermediate Targets for R1-R6 Growth Cones

R1-R6 axons select their targets in two temporally distinct steps: initial targeting to lamina glia during larval development (Figure 2A) and selection of appropriate target neurons during pupation (Figure 2C). Genetic ablation experiments demonstrate that lamina glia provide a stop signal to incoming R1-R6 growth cones (Poeck et al., 2001). In wild-type animals, R cell axons terminate between two layers of lamina glia. In mutants in which glia cells do not migrate into the target region, the vast majority of R cell axons fail to terminate in the lamina and project into the underlying medulla (Poeck et al., 2001; Suh et al., 2002). This implies that a glia-derived factor(s) directs R1-R6 growth cones to terminate in the lamina. The use of lamina glia as intermediate targets allows R cell axons to enter the target field prior to the differentiation of their postsynaptic partners. This enables R cell axons to use induction of cell division in lamina neuronal precursors as a means of matching the number of postsynaptic units to afferents. That is, when R cell axons enter the target field, their lamina neuronal targets are not yet differentiated. This use of transient targets appears conceptually analogous to the role of synaptic connections between thalamocortical axons and a transient cell population (i.e., subplate neurons) prior to the formation of specific cortical layers (Ghosh et al., 1990). Transient targets may provide a general developmental strategy for coordinating the timing of histogenesis between different brain regions with the formation of connections between them.

Pharmacological studies suggest that nitric oxide plays a role in maintaining R1-R6 growth cones within the lamina (Gibbs and Truman, 1998). In wild-type animals, R1-R6 growth cones remain between layers of lamina glia for about 36 hr before extending to their neuronal targets. While disruption of nitric oxide signaling does not affect the initial targeting of these growth cones to the lamina, it does cause R1-R6 growth cones to inappropriately invade the medulla during pupal development.

Afferent-Afferent Interactions Regulate R1-R6 Specificity

R1-R6 growth cones undergo a complex rearrangement during pupal development to innervate their appropriate lamina targets (Meinertzhagen and Hanson, 1993; Clandinin and Zipursky, 2000). Initially, R1-R6 growth cones from the same ommatidium extend into the lamina as a single fascicle, their growth cones terminating in a tight cluster nestled between lamina glia. During pupal development, each growth cone extends out of this cluster to innervate a different column of lamina target neurons lying in invariant positions with respect to the ommatidial bundle (Figure 2C). As a result, six R1-R6 axons from a single ommatidium innervate a characteristic pattern of six different targets oriented with respect to the dorsoventral and anteroposterior axes of the lamina target field. Remarkably, this precise, asymmetric connectivity pattern arises even though the array of lamina target neurons is arranged in a spatially symmetric pattern around each ommatidial bundle. That is, the only distinguishing feature of different targets for the R1-R6 neurons from a single ommatidium is their precise position relative to the bundle.

What are the cellular and molecular mechanisms that underlie this rearrangement? Early electron microscopic observations led to the notion that afferent-afferent interactions would play a prominent role in this process (Meinertzhagen and Hanson, 1993). During early pupal development, a precise sequence of contacts between R cell growth cones that correlate with changes in the orientation of each growth cone was described. At the end of this sequence, each growth cone is oriented toward its synaptic partner prior to axon extension across the lamina surface.

Genetic ablation of specific R1-R6 subtypes demonstrated that interactions among them, indeed, play critical roles in determining the pattern of targets chosen (Clandinin and Zipursky, 2000). In particular, R3 and R4 appear to play a dominant role in controlling R cell target choice; in the absence of R3 and R4, the remaining four R1-R6 cells frequently choose inappropriate targets. By contrast, removing R1 and R6 had no effect on the projections of R3 and R4. This argues for specific interactions between R cell growth cones within the same or neighboring bundles. Genetic mosaic experiments demonstrate that the orientation of the projection pattern is largely, though not entirely, independent of target-derived factors (Clandinin and Zipursky, 2000). Rather, projection orientation is predominantly determined by the orientation of the R cell axon bundle as it enters the target field. These experiments suggest that much of the information that determines the R cell connectivity pattern in the lamina is borne by the afferent axons themselves and decoded by interactions between them. Implicit in this view is the notion that the regulation of cell surface molecules that mediate such interactions will prove critical in directing R1-R6 target choice.

Recent genetic studies suggest that interactions between R1-R6 may also play a crucial role in regulating the initial targeting to the lamina. Banerjee and coworkers discovered that the Runt transcription factor is selectively expressed in R7 and R8 (Kaminker et al., 2002). Based on this pattern, they speculated that Runt may control the expression of genes regulating targeting to the medulla. Though loss-of-function *runt* mutations did not lead to mistargeting of R7/R8 growth cones, perhaps due to redundancy with other Runt proteins, misexpression of Runt in R1-R6 neurons redirected their growth cones to terminate in the medulla. As Runt did not otherwise alter R1-R6 differentiation, it was speculated that Runt controls the expression of genes regulating target recognition. Runt expression in R2 and R5 was shown to be sufficient to retarget all R1-R6 neurons to the medulla. Hence, during normal development, interactions between R2 and R5 axons with the surrounding R1, R3, R4, and R6 axons play a crucial role in targeting to the lamina.

The role of afferent-afferent interactions and fascicle

structure in influencing target selection has also been examined in the context of vertebrate retinal ganglion cell projections to their targets in the brain. In these systems, it is clear that axons can still project to their appropriate targets despite experimental disruptions in fascicle structure. This type of experiment has given rise to the notion that axon/axon interactions do not play a prominent role in target specificity. Nevertheless, several observations argue for the importance of these interactions. Using a function-blocking antibody to reduce the activity of the Neural Cell Adhesion Molecule (NCAM) in the chick (which has an optic nerve of precise structure), axon fasciculation patterns were disrupted in the optic nerve, and the specificity of targeting within the tectum was assessed (Thanos et al., 1984). In these experiments, axons that extended early in the innervation process frequently fasciculated inappropriately and followed aberrant pathways through the tectum but, upon contacting the tectal surface, frequently selected topographically appropriate targets. In contrast, later developing axons that did not encounter the surface of the tectum innervated topographically inappropriate targets after making fasciculation errors. One parsimonius interpretation of these results is that target selection in the tectum is determined by both fascicle structure and by target-derived cues; when fascicle structure is disrupted, axons can still respond, through short-range interactions, to cues on the surface of the tectum that direct axons toward their appropriate targets. Late-developing fibers that do not encounter the surface of the tectum do not respond to these signals and are directed toward their targets largely by their position within the fascicle. In contrast, in higher vertebrates such as rats, the optic nerve lacks stereotyped structure, and the initial retinal ganglion cell projection to the superior colliculus is imprecise (Simon and O'Leary, 1990; Simon and O'Leary, 1991). In these systems, selection of topographically appropriate targets occurs through a process involving selective elimination of inappropriate connections (O'Leary et al., 1986). Recent studies suggest that the selection of the appropriate targets within the superior colliculis also requires competitive interactions, either direct or indirect, between different populations of retinal ganglion cell axons expressing different levels of EphA receptors (Brown et al., 2000).

Together, these findings suggest that, in addition to the cellular recognition mechanisms regulating interactions between growth cones and their targets, interactions between afferents also play an important role in contributing to specificity.

Histological Screens in Genetically Mosaic Animals Identified Genes Required for R Cell Axon Guidance and Targeting

R cells are highly amenable to genetic manipulation. This is due to the intensive study of pattern formation and cell fate determination in the developing eye and the availability of an impressive battery of genetic techniques in *Drosophila*. These techniques allow one to specifically manipulate R cell genotypes through both gain- and loss-of-function studies (Figure 3) in an otherwise wild-type animal (Newsome et al., 2000a; Stowers and Schwarz, 1999; Lee et al., 2001). Markers for different classes of axons facilitate both genetic screening for targeting mutants and phenotypic analyses. That different R cells mediate different visual behaviors forms the basis of genetic screens for mutations disrupting patterns of R cell connections.

Initial efforts to identify the molecular mechanisms regulating the formation of R cell connections applied histological screening methods to identify mutations disrupting them. Early screens using paraffin sectioning and conventional histological stains (Heisenberg, 1980; Ebens et al., 1993; Cheyette et al., 1994) were laborious and identified mutations that largely affected connection formation indirectly. Subsequent genetic screens utilized histological methods in the third larval stage to assay R cell axons as they project to their targets. These screens focused on genes whose functions were required in R cells and whose mutant phenotypes did not affect R cell fate specification (Martin et al., 1995; Garrity et al., 1996).

These approaches were significantly limited, as genes required for axon guidance are likely to function not only in R cells but also in developmental processes essential for viability. As such, these mutants would not have survived to sufficiently late stages of development to be assessed for their effects on R cell connectivity. To address this problem, Dickson and colleagues (Newsome et al., 2000a) developed an elegant method for generating eyes entirely homozygous for a randomly mutagenized chromosome in an otherwise normal animal (i.e., a heterozygous animal) by targeting mitotic recombination selectively to the eye primordium (Figure 3A). In this way, mutant R cell axons were visualized projecting into wild-type optic ganglia. This approach led to the identification of a large collection of mutations affecting the pattern of R cell projections during initial targeting events in larval development (Newsome et al., 2000a). Treisman and colleagues also used this genetic method to screen for defects in adult R cell projections using a histological approach (see below) (Maurel-Zaffran et al., 2001).

These histological screens identified several genes that affect targeting of R cell axons to the lamina. *brakeless* mutants recapitulate the severe mistargeting phenotype seen in backgrounds that lack lamina glia or in which Runt is misexpressed in R2 and R5 (Senti et al., 2000; Rao et al., 2000). The *brakeless* gene encodes a nuclear protein. Interestingly, the transcription factor Runt, which is normally expressed in R7 and R8, becomes derepressed in R2 and R5 in *brakeless* mutants (Kaminker et al., 2002). As discussed above, misexpression of Runt in these two R cells is sufficient to direct all R1-R6 axons to target inappropriately in the medulla. The mistargeting in *brakeless* is not associated with transformation of other features of R1-R6 cells into R7/R8 neurons. These findings suggest that *brakeless* and *runt* act in a pathway controlling genes regulating signaling systems in the growth cone that recognize targeting determinants produced by lamina glia.

While mutations in *brakeless* give rise to strong targeting defects in R1-R6 neurons, mutations in genes encoding cell surface proteins that give rise to defects of similar strength have not been identified. Mutations

in the receptor tyrosine phosphatase *PTP69D* cause a comparatively weak R1-R6 mistargeting phenotype (Garrity et al., 1999; Senti et al., 2000). *PTP69D* is expressed in R cell axons and growth cones and is required for guidance of many neurons in the developing embryonic central nervous system (Desai et al., 1996, 1997). Incomplete mistargeting may reflect the existence of redundant signaling pathways that promote R1-R6 termination in the lamina. Alternatively, PTP69D may only modulate the activity of an as yet unidentified receptor for an R1-R6 targeting signal, much as the protein tyrosine phosphatase CD45 promotes the signaling pathway activated by engagement of the T cell receptor in the vertebrate immune system (see Hermiston et al., 2002).

These screens also identified mutations affecting an evolutionarily conserved signal transduction pathway in R cell growth cones. These include *dreadlocks* (*dock*), *p21 activated protein kinase* (*Pak*), and *Trio* (Garrity et al., 1996; Hing et al., 1999; Newsome et al., 2000b). Mutations in these genes result in complex phenotypes with highly disorganized projections into the optic ganglia, including abnormalities in both local topographic mapping and in target specificity. *dock* encodes the fly homolog of Nck, an SH3/SH2 adaptor protein. *Pak* encodes a kinase that binds to Dock and regulates the actin cytoskeleton downstream from the activated Rho family GTPases Cdc42 and Rac. *Trio* encodes a Rho family guanine nucleotide exchange factor that activates Rac. Genetic and biochemical experiments suggest that Dock and Trio act in parallel to regulate Pak activity in R cell growth cones (Newsome et al., 2000b), though the specific receptor systems that act upstream of them in R cells are not known (see below). While *Trio* mutations share some features in common with *dock* and *pak*, they also give rise to general defects in axon outgrowth. Studies in a simple embryonic nerve projection, Bolwig's nerve, suggest that Dock and Pak (the role of Trio in this system has not been assessed) act in a signal transduction pathway promoting an attractive response to an intermediate target (see below; Schmucker et al., 2000). Recent studies also suggest that Nck and Pak act downstream from the receptor DCC in regulating attractant response to a gradient of netrin in vertebrate growth cones (E. Stein and M. Tessier-Lavigne, personal communication), suggesting that this may represent part of an evolutionarily conserved pathway regulating growth cone attraction.

Biochemical studies were pursued to identify receptors that lie upstream of Dock in axon guidance. Dock-associated proteins were isolated using a cell culture-based approach (Schmucker et al. 2000; J. Clemens, personal communication). One of these was homologous to an Ig superfamily member in mammals, called Down Syndrome Cell Adhesion Molecule or Dscam (Yamakawa et al., 1998). Dscam maps to a region of chromosome 21 in humans and may contribute to the mental retardation associated with Down Syndrome. In *Drosophila*, genetic and biochemical evidence supports the view that Dscam, Dock, and Pak act within Bolwig's nerve growth cones to promote interactions between the growth cone and an intermediate target (Schmucker et al., 2000). Preliminary studies indicate that Dscam is required for both R1-R6 and R7 connections (T. Hummel, L. Vasconcelos, I.A. Meinertzhagen, and S.L.Z., unpublished data). As these phenotypes are different from Dock and Pak and largely occur at later stages of target selection, it seems likely that Dock and Pak act downstream of other receptors earlier in visual system development. Based on genetic and biochemical studies, Pick and colleagues have proposed that Dock and Pak act downstream of the *Drosophila* insulin receptor at early stages of R cell axon guidance (J. Song and L. Pick, submitted). As we discuss below, *Drosophila* Dscam exhibits extraordinary diversity generated through alternative splicing, and we speculate that this may play an important role in the specificity of cellular recognition underlying connection specificity.

In summary, genetic screens using histological-based methods in developing tissue have led to the identification of genes required for normal R cell connectivity and have provided insights into the molecular mechanisms controlling growth cone guidance. These studies have yet to provide a molecular picture of the strategy these neurons use to select between the lamina and the medulla. In contrast, both histological and behavioral screens in adult tissue have led to the identification of genes encoding cell surface proteins required for R1-R6 and R7 neurons to select specific targets once within the appropriate target layer.

Behavioral Screens Have Identified Genes Regulating R Cell Target Choice: N-Cadherin and Lar Regulate R1-R6 and R7 Targeting

Two behavioral approaches have been applied to the isolation of R cell targeting mutants (Lee et al., 2001; Clandinin et al., 2001). Each has two components: a behavioral assay specific to the R cell subtype of interest and a genetic mosaic method for generating flies in which R cells or a subclass of them are rendered homozygous for a mutagenized chromosome using targeted mitotic recombination. Mutations affecting R1-R6 connectivity were identified in screens in which all R cells were homozygous for the mutagenized chromosome (Figure 3A), and flies were tested in an optomotor paradigm. By targeting mitotic recombination to only a small subset of R cell precursors, R7 neurons were selectively rendered homozygous, in an otherwise heterozygous animal, and tested in a behavioral paradigm in which flies select between visible and ultraviolet light (T. Herman and S.L.Z., unpublished data; Lee et al., 2001; Figure 3B). This scheme was adapted to the MARCM technique (Lee and Luo, 1999) to visualize mutant R7 connections in an otherwise wild-type animal (Figure 3B). This, in a sense, represents single-cell genetics in the context of a multicellular organism. After identifying a set of behavioral mutants, the development of R cells and the connections elaborated by different classes of them are assessed to identify the connectivity mutants within the collection.

Mutations affecting N-cadherin were identified in screens for R1-R6 and R7 targeting (Lee et al., 2001). N-cadherin is a classical cadherin (Figure 4A). Like other classical cadherins it possesses a cytoplasmic domain that physically interacts with β-catenin and, though its extracellular domain differs considerably from verte-

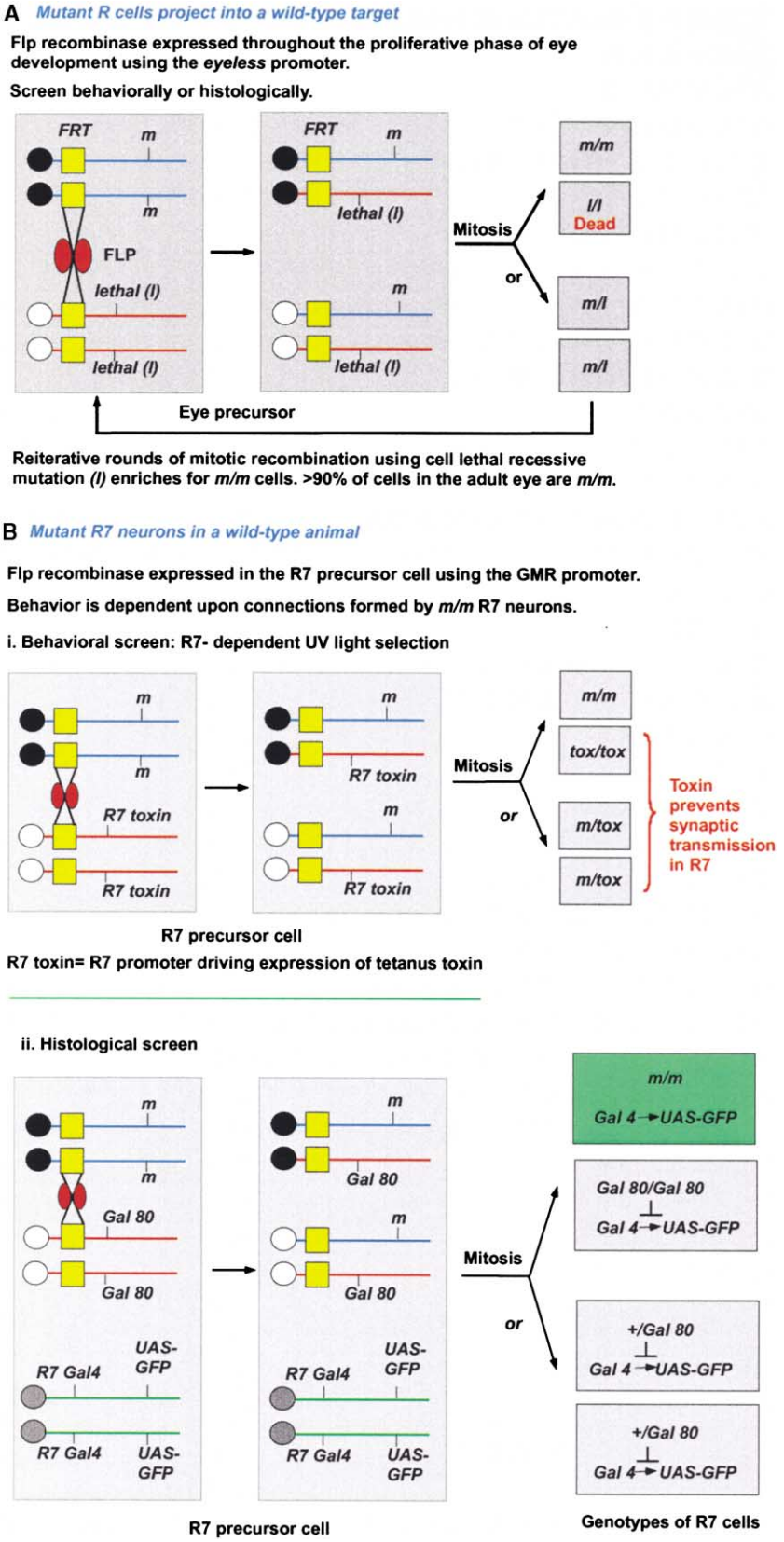

Figure 3. Genetic Mosaic Screens for R Cell Connectivity Mutants

(A) Generation of eye-specific mosaics by targeting FLP recombinase expression to the eye primordium. Mitotic recombination is directed to eye precursor cells using the FLP/FRT recombination method. The *eyeless* promoter drives FLP expression in the eye primordium at an early stage and persists through the proliferative phases of eye development. As such, reiterative rounds of mitotic recombination occur. By

brate N-cadherin, it too mediates homotypic cell adhesion. N-cadherin plays multiple roles in regulating axon guidance in the embryo (Iwai et al., 1997). N-cadherin is widely expressed in R cell axons and their target neurons in the lamina and the medulla. While removal of N-cadherin from all R cells gives rise to a complex phenotype arguing for multiple roles in regulating R cell connectivity, selective removal from single R1-R6 or R7 neurons in an otherwise wild-type background, gives rise to discrete targeting defects, supporting a role for N-cadherin in the cellular interactions mediating target selection.

N-Cadherin in R1-R6 Targeting

In genetic mosaics in which all R cells are rendered homozygous for N-cadherin, R1-R6 mutant axons target to the lamina plexus but only infrequently extend from the ommatidial bundle to their appropriate targets. Indeed, single mutant R1 or R6 neurons surrounded by wild-type R cell axons and lamina neurons often fail to extend to the appropriate synaptic partner. They do, however, elaborate the characteristic morphology of the presynaptic terminal within a spatially inappropriate cartridge (Clandinin et al., 2001) (Figure 4B). This result indicates that N-cadherin is required cell autonomously to regulate R1-R6 cell target choice.

N-cadherin could mediate interactions between R cell growth cones and lamina target neurons. Alternatively, N-cadherin may mediate interactions between R cell growth cones within the fascicle or between R cell growth cones in adjacent fascicles to orient growth cones toward their targets prior to extension. In this view, N-cadherin facilitates specific interactions between R cells that then lead to their precise orientation toward their targets. Based on the characterization of the developmental phenotypes associated with R7 targeting defects in *N-cadherin* mutants (see below), we favor the view that N-cadherin is required for interactions between afferents and their targets.

Ultrastructural analysis of synapses in viable flies bearing weak alleles of *N-cadherin* (i.e., partial loss-of-function in all afferents and target neurons) demonstrated that synapses formed between R1-R6 neurons and their lamina target neurons are frequently morphologically aberrant (Iwai et al., 2002). This raises the possibility that N-cadherin also plays a role in synapse assembly or maintenance. Given the strong effects of N-cadherin mutations on R cell target choice, however, it remains possible that this synaptic phenotype is an indirect consequence of earlier defects.

N-Cadherin in R7 Targeting

Loss of N-cadherin activity from R7 growth cones causes precise targeting defects (Lee et al., 2001; Clandinin et al., 2001). In wild-type animals, R8 axons project to a superficial layer of the medulla. Some 12 hr later, each R7 axon follows the R8 axon from the same ommatidium and extends some 2–3 μm past the R8 terminus to a deeper layer in the medulla (see Figure 2B). In adult mosaic animals in which a fraction of R7 cells are made homozygous mutant in an otherwise wild-type background, *N-cadherin* mutant R7 axons terminate inappropriately in the R8 recipient layer (Figure 4C). One possibility is that the temporal sequence of innervation is important in regulating targeting of R7 to the deeper layer of the medulla. Perhaps R8 growth cones physically prevent later-arriving R7s from innervating the R8-recipient layer. Mutant phenotypes, however, suggest that the decision of the R7 axon to bypass the R8 layer and to terminate within the R7-recipient layer represents a discrete choice point in R7 target selection. That is, developmental analysis in N-cadherin mutants demonstrated that the mistargeting phenotype is caused by the failure of R7 axons to form stable contacts within the R7-recipient layer. In particular, *N-cadherin* mutant R7 axons initially extend beyond the R8 layer early in development but then retract back to the R8 layer as development proceeds (C.-H. Lee and S.L.Z., unpublished data). As N-cadherin is expressed on both R7 growth cones and processes in the R7 targets, it is likely then that homotypic N-cadherin interactions mediate adhesion between them.

Lar in R1-R6 and R7 Targeting

Mutations in the gene encoding Lar (homologous to human leukocyte homology antigen-related receptor tyrosine phosphatase), were also identified in behavioral screens for defects in both the R1-R6 and R7 neurons (Clandinin et al., 2001) and in histological screens for defects in R7 connectivity (Maurel-Zaffran et al., 2001). Indeed, the phenotypes of *Lar* mutant R1-R6 neurons and R7 neurons projecting into a wild-type target are largely indistinguishable from *N-cadherin* mutants. Lar

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including a recessive cell-lethal mutation [*lethal* (*l*)] on the nonmutagenized chromosome, the reiterated rounds of recombination selectively enrich for cells homozygous for the mutagenized chromosome (*m*/*m*). These flies can be screened either histologically (see Newsome et al., 2000a) or behaviorally (Clandinin et al., 2001). As FRT-mediated mitotic recombination can only be targeted to a single chromosome arm at a time, screens must be done separately for each arm.

(B) Generation of R7-specific mosaics by targeting FLP recombinase to R7 precursor cells. FLP recombinase is expressed in the last cell division in the developing eye under the control of the GMR promoter (Lee et al., 2001). This division gives rise to all R7 cells (as well as R1 and R6 cells). R2-R5 and R8 cells are generated from the preceding cell division and are, therefore, heterozygous. Mutations disrupting R7-dependent function can be identified through (1) behavioral or (2) histological screens. (1) The R7 behavioral screen: the inclusion of a transgene on the nonmutagenized chromosome in which an R7-specific promoter drives tetanus toxin (R7-toxin) ensures that the only synaptically active R7 cells in the eye are those that are homozygous for the mutagenized chromosome; tetanus toxin cleaves synaptobrevin, thereby inactivating synaptic transmission. Hence, the R7-dependent behavior is mediated solely by R7 cells homozygous for the mutagenized chromosome. (2) The R7 histological method: homozygous mutant R7 neurons can be selectively labeled using the MARCM technique (Lee and Luo, 1999; Lee et al., 2001). The inclusion of the Gal-80 gene driven by the tubulin promoter on the nonmutagenized chromosome ensures that the only labeled cells are those homozygous for the mutagenized chromosome. The cells are labeled in a bipartite system in which two transgenes are required to label mutant R7 neurons: an R7 promoter driving Gal4 and a UAS promoter driving synaptobrevin-GFP (or another synaptic or axonal marker), which in this example are inserted onto a different chromosome. Gal 80 is a transcriptional repressor that inhibits the transactivator Gal4.

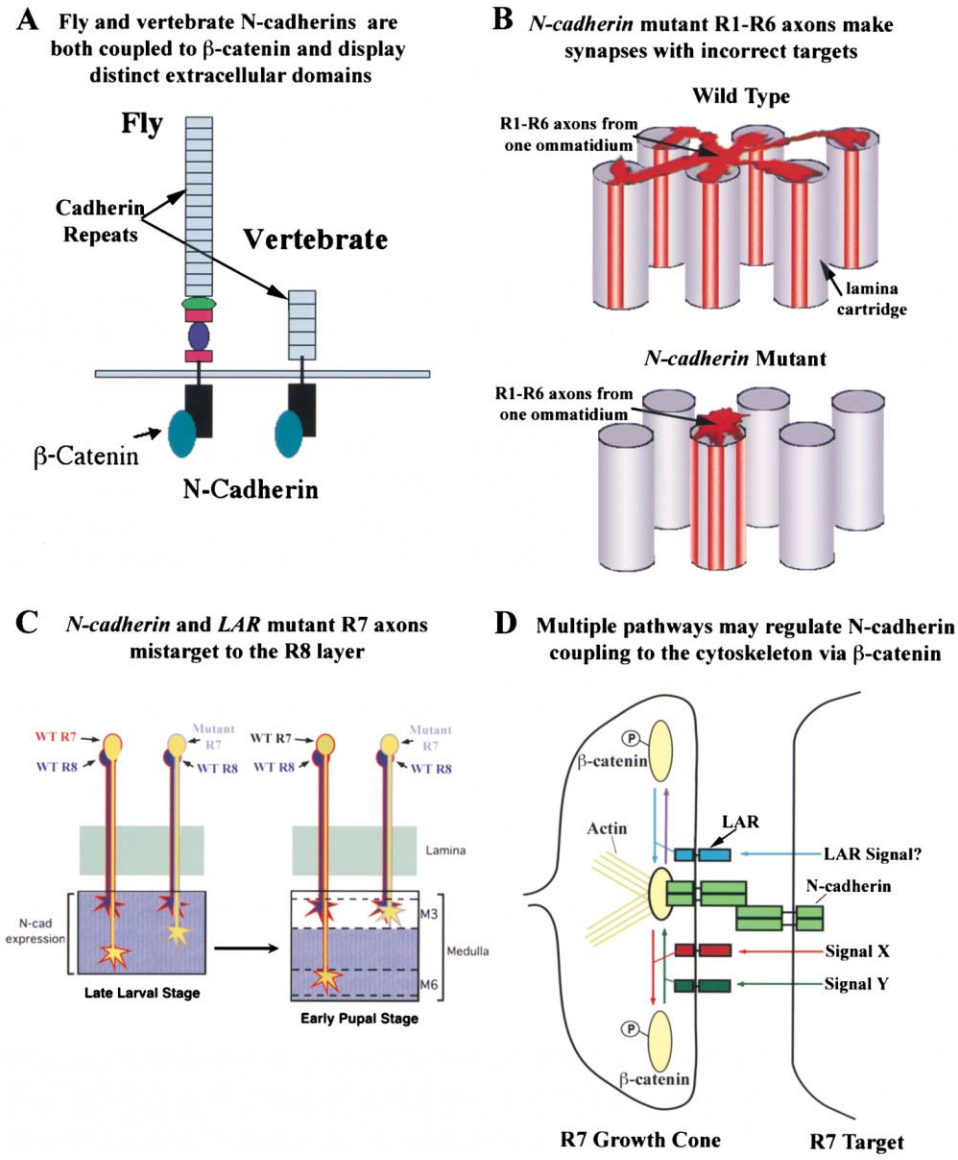

Figure 4. N-Cadherin Regulates R Cell Targeting

(A) Fly and vertebrate N-cadherin have a similar cytoplasmic domain and physically interact with β-catenin. Although these proteins have significantly different extracellular domains, they both function as homotypic cell adhesion molecules. In addition to the cadherin repeats, fly N-cadherin also comprises other extracellular domains characteristic of invertebrate classic cadherins.

(B) N-cadherin is required in a cell-autonomous fashion to control the targeting of R1-R6 axons to specific postsynaptic units (cartridges) in the lamina. The figure presents a view of the terminal region of a fascicle of axons from a single ommatidium at the surface of the lamina neuropil. R1-R6 neurons project away from each other and to distinct targets. In *N-cadherin* mutants, however, they frequently fail to extend across the surface of the lamina and instead project down into the cartridge directly beneath the fascicle.

(C) In wild-type, R7 growth cones project past the R8 growth cone into a deeper layer in the medulla. R7 mutant axons lacking either N-cadherin or Lar, in an otherwise wild-type background, project past R8s. In some cases, they extend into the R7-recipient layers, and other cases fall short. During pupal development, these mutant R7 axons retract to the R8 layer.

(D) We propose that N-cadherin promotes adhesion between growth cones and their targets, here shown for R7 and its target in the medulla. β-catenin phosphorylation is an important regulatory step in N-cadherin-based adhesion systems. In this context, Lar may promote adhesion by dephosphorylating β-catenin. We envision that the N-cadherin adhesive system may be strengthened or weakened by other signals. This dynamic interplay between multiple signaling systems may provide a robust and flexible system for generating multiple targeting specificities.

could regulate N-cadherin interactions (Figure 4D), or alternatively, it could act in a pathway parallel to it. Biochemical studies in vertebrate systems are consistent with Lar acting in conjunction with N-cadherin. N-cadherin and Lar form a complex, in part, due to their association with β-catenin (Kypta et al., 1996). As tyrosine phosphorylation negatively regulates the association of β-catenin with cadherins (Kypta et al., 1996; Brady-Kalnay et al., 1998), Lar may promote adhesion by dephosphorylating β-catenin. This is supported by

the observation that RPTPμ, another receptor tyrosine phosphatase, stimulates N-cadherin-dependent outgrowth (Burden-Gulley and Brady-Kalnay, 1999).

Alternatively, Lar could act in parallel to N-cadherin. This may occur through its specific binding to a target-derived signal distinct from N-cadherin; indeed, vertebrate Lar has been shown to bind to a laminin-nidogen complex (O'Grady et al., 1998). In this way, Lar would provide a second adhesive mechanism to stabilize the interaction of the R7 growth cone and its target in parallel to homotypic cell surface interactions mediated by N-cadherin. Transmission to the actin cytoskeleton may proceed through interactions with β-catenin, or alternatively, Lar may act through distinct intracellular signaling pathways. Indeed, both gain- and loss-of-function mutations in the actin regulatory proteins Ena and Trio, a Rho family guanine nucleotide exchange factor, have been shown to modify the Lar R7 targeting phenotype (Maurel-Zaffran et al., 2001). Loss-of-function studies also indicate that the receptor tyrosine phosphatase PTP69D plays a role in controlling layer-specific targeting of R7 growth cones (Newsome et al., 2000a); in an entirely mutant eye, PTP69D mutant R7 axons also terminate at the R8 recipient layer. It is not yet known whether this is a cell-autonomous function like that of Lar and N-cadherin.

Cadherins May Be Conserved Determinants of Connection Specificity

We favor the view that cadherin-based adhesion is an essential component of the cell surface interactions that mediate formation of connections between many different types of neurons. We propose that the specificity of these interactions is regulated by (1) modulation of cadherin adhesivity via various posttranslational mechanisms and (2) combinatorial interactions with other cadherins, cadherin isoforms, and other families of diverse cell surface recognition molecules.

Cadherin function may be dynamically regulated within different visual system growth cones to specify the formation of distinct connections. We envision that modulation of N-cadherin activity plays a key role in R7 targeting. N-cadherin is required in R7 for growth cones to execute a simple choice between two immediately adjacent layers in the medulla, the R7 and R8 recipient layers. The precise temporal and spatial pattern of R cell development and R cell axon guidance reduces the demand for a highly selective mechanism underlying recognition between the growth cone and its target. Differences in activation of Lar between R7 and R8 may provide the crucial distinction between these two layers. In this view, differential modulation of N-cadherin, β-catenin, or additional components of the adhesion complex via tyrosine phosphorylation allows R7 growth cones to extend beyond R8 to make stable connections in the R7 recipient layer. As overexpression of Lar does not promote R8 mistargeting to the R7 recipient layer, other regulatory mechanisms must also contribute to layer specificity. While it is conceivable that modulation of N-cadherin activity is a primary determinant of R7 targeting, it seems likely that additional specificity determinants will be required to determine the more complex and intricate pattern of connections between R1-R6 neurons and their lamina targets. Indeed, we have recently observed that R1-R6 but not R7 specificity relies on the activity of Flamingo, a cadherin-related cell surface receptor (R. Lee, T.R.C., and S.L.Z., unpublished data).

It is important to emphasize that the analysis in connection specificity for R1-R6 and R7 that we have described here is in the context of selecting the appropriate group of cells or processes (either a layer in the medulla or a cartridge in the lamina); once within these regions, highly specific mechanisms must underlie the selective recognition between different neuronal cell surfaces involved in synapse formation. For instance, while targeting to the R7-recipient layer may be relatively simple, R7 neurons only form synaptic connections at a specific layer within the medulla neuropil some time later in development, although their axons are in close proximity to processes of many different lamina and medulla neurons within a column (i.e., axons and dendrites of some 50 different neurons) in the medulla neuropil. Regrettably, the fine structure of R7 synapses and the identity of postsynaptic processes is not yet known. In the lamina, however, where connection specificity has been described at high resolution through electron microscopic reconstruction experiments, R1-R6 neurons form complex synapses, called tetrads, with a precise geometric relationship between a single presynaptic and a set of four postsynaptic terminals. Hence, it seems likely that the specificity of neuronal connections relies on other cell surface labels or combinations of them in addition to N-cadherin.

There are intriguing parallels between the functions for N-cadherin in the fly visual system and for classical cadherins in the vertebrate nervous system. Immunolocalization studies led to the proposal that classical cadherins play important roles in both the establishment and maintenance of synaptic structures, as well as in the choice of synaptic partner as part of a synaptic code (for example, see Fannon and Colman, 1996; Shapiro and Colman, 1999). In these studies, specific patterns of cadherin expression at mature synapses were used to infer a role in synapse formation, maintenance, or function. Antibody disruption experiments both in vivo and in vitro support the view that N-cadherin is required for the formation or stabilization of connections of retinal ganglion cells to their appropriate layers within the tectum (Inoue and Sanes, 1997). These studies, however, leave open the precise developmental stages which require N-cadherin activity. As we have described for *Drosophila* N-cadherin, it is possible that vertebrate classical cadherins are required both for neurons to select the appropriate cell within the target field, as well as to maintain the structure of the mature synapse. Modulation of cadherin activity may also be important in regulating connectivity in vertebrate systems. For example, tyrosine phosphorylation (Matsuyoshi et al., 1992) and synaptic activity (Tanaka et al., 2000) have been shown to regulate the adhesive and molecular properties of classical cadherins. Indeed, dynamic changes in cadherin function are likely to play important roles in regulating the structural and morphological changes in the synapse associated with synaptic plasticity (Togashi et al.,

2002; Murase et al., 2002; Tang et al., 1998). Further developmental and mosaic analyses are likely to reveal additional functional similarities between the vertebrate and invertebrate classical cadherins.

Molecular Diversity May Contribute to Connection Specificity

How many different cell surface recognition proteins determine the pattern of connections between neurons? Presumably, the answer to this question reflects the ratio of appropriate choices to inappropriate ones within the target region accessible to a given growth cone. That is, as the number of inappropriate targets decreases, less information is necessary to determine the pattern of connections. Indeed, the stepwise process of axon guidance in some systems ensures that once growth cones are in the target field they encounter only a limited set of potential targets.

Complexity in neuronal connectivity patterns may rely in some systems on families of cell surface receptors of rather limited diversity that act through combinatorial association. For example, the *Drosophila* genome encodes three classical cadherins, all of which are expressed in the visual system (Lee et al., 2001; Tepass et al., 1996; C.-H. Lee and S.L.Z, unpublished data), and N-cadherin is found in alternative forms in the developing eye disc through alternative splicing (C.-H. Lee, A. Chiba, H. Robertson, and S.L.Z., unpublished data). In the mouse, there are approximately 20 different classical cadherins (Uemura, 1998), many of which are expressed in the nervous system (Suzuki et al., 1997). The increased complexity of the classical cadherin family in vertebrates as compared to the fly may reflect the increased complexity of neuronal connectivity patterns. In addition to classical cadherins, numerous Ig-containing cell adhesion proteins and integrins may play prominent roles in connection specificity. In vertebrates, several families of cell surface proteins, each containing some 20 to 50 different members, have been identified as candidates for controlling connection specificity. These include, in addition to the classical cadherins, cadherin-related neuronal receptors (Kohmura et al., 1998; Wu and Maniatis, 1999) and the MHC class I proteins (Huh et al., 2000). A combination of rather general guidance signals and cell adhesion molecules has also been shown to play a role in motorneuron targeting in the *Drosophila* embryo (Winberg et al., 1998). The combinatorial use of different cell surface receptors could, in principle, provide an extensive recognition code.

Compelling evidence for the importance of molecular diversity in specifying neuronal connectivity has come from studies in the vertebrate olfactory system. Axel and colleagues have shown that the 1000 or more odorant receptors, members of the seven-transmembrane superfamily, play an instructive role in connection specificity (Wang et al., 1998). The 5 million olfactory neurons in the mouse can be divided into approximately 1000 different classes based on the expression of odorant receptors. Each olfactory neuron expresses only a single receptor, and those expressing the same receptor form connections with the same group of postsynaptic cells arranged into clusters called glomeruli. Knock-out of individual receptors disrupts targeting, while knock-in experiments in which the gene encoding one receptor replaces the coding sequence of another resulted in targeting to a novel site different from both the replacement receptor and the receptor being replaced. Hence, odorant receptors play an instructive role in targeting though, clearly, this alone is not sufficient to determine connection specificity. As odorant receptors are expressed specifically in olfactory neurons, it remains unclear whether similar mechanisms will regulate projection specificity in other regions of the nervous system.

The *Drosophila* genome encodes at least one example of a cell surface receptor that displays remarkable molecular diversity (Schmucker et al., 2000). The Dscam protein is a single-pass transmembrane protein comprising ten immunoglobulin domains, six fibronectin type III repeats, and a cytoplasmic domain with multiple binding sites for SH3 and SH2 domains. Through alternative splicing of a set of highly related exons, the *Dscam* gene can give rise to as many as 38,016 different proteins, each with the same domain structure, that differ in their amino acid sequences within the extracellular and transmembrane domains. A vast array of these isoforms is expressed in both the developing embryonic and postembryonic nervous systems. Dscam regulates axon pathfinding in the embryo, at least in some contexts, in a pathway requiring Dock and Pak (Schmucker et al., 2000; see above) and is required for correct targeting, branching, and dendritogenesis of a variety of neurons in the adult central nervous system (Wang et al. 2002; T. Hummel, L. Vasconcelos, X. Zhan, and S.L.Z., unpublished data). Preliminary studies suggest that Dscam, also, is required in R cell growth cones for normal synapse formation (T. Hummel, L. Vasconcelos, I.A. Meinertzhagen, and S.L.Z., unpublished data). The function of different isoforms in regulating connection specificity is not known. Nevertheless, the multiplicity of Dscam isoforms raises the possibility that alternative splicing may be a common strategy to generate extensive molecular complexity on the cell surface and, hence, multiple cell surface labels from a relatively small number of genes.

While Dscam diversity has been widely conserved in insects (J. Clemens, B. Gravely, and S.L.Z., unpublished data), alternative splicing does not generate diversity in the mammalian Dscams (the two identified in mouse and human) (Yamakawa et al., 1998; Agarwala et al., 2001); interestingly there are three additional Dscams in *Drosophila*, none of which show extensive diversity either (J.C. Clemens and S.L.Z., unpublished data). While it is possible that other Dscams exhibiting diversity remain to be discovered in the mouse and human genome, it is possible that molecular diversity evolved in other genes or gene families in vertebrates. Hence, diversification of receptor structure and recognition may have evolved independently in different lineages through gene duplication and sequence divergence or through the utilization of posttranscriptional mechanisms such as alternative splicing or RNA editing.

Concluding Remarks

We favor the view that a cadherin-based adhesion system lies at the molecular core of target recognition (Fannon and Colman, 1996). Indeed, this role for cadherins

may have evolved from an evolutionarily ancient adhesive function in epithelia (Kelly, 1988). We believe that the problem of target specificity has been solved independently in a variety of different anatomical contexts and that these solutions reflect the constraints imposed by the neuronal architecture, behavioral function of the circuit, and their evolution. These mechanisms may utilize precise spatial and temporal control of growth cone-target interactions or may involve the molecular matching of determinants through diversification of cadherins, combinatorial interactions between different sets of cell surface proteins, and molecular diversity of cell surface proteins. The extraordinary complexity of the cellular environment confronting cells in the central nervous system makes unraveling the molecular basis of synaptic specificity particularly challenging. The single-cell genetic methods available in the fly visual system provide a means to systematically dissect these mechanisms. A comparative approach that extends single-cell analysis to targeting in other invertebrate and vertebrate contexts promises to provide insight into the range of mechanisms utilized to determine synaptic specificity.

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