Leading Edge
Review

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Chemoaffinity Revisited: Dscams, Protocadherins, and Neural Circuit Assembly

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The chemoaffinity hypothesis for neural circuit assembly posits that axons and their targets bear matching molecular labels that endow neurons with unique identities and specify synapses between appropriate partners. Here, we focus on two intriguing candidates for fulfilling this role, *Drosophila* Dscams and vertebrate clustered protocadherins (Pcdhs). In each, a complex genomic locus encodes large numbers of neuronal transmembrane proteins with homophilic binding specificity, individual members of which are expressed combinatorially. Although these properties suggest that Dscams and Pcdhs could act as specificity molecules, they may do so in ways that challenge traditional views of how neural circuits assemble.

Introduction

Two experiments have had a decisive influence on our ideas about how neurons form the complex patterns of synaptic connections that underlie mental activities. Both were performed long ago, relied on simple behavioral assays, didn't involve molecules, and focused on regeneration following nerve injury in adults rather than development.

In the first, John Langley (Langley, 1895) analyzed regeneration in the autonomic nervous system of a cat. He had found that axons from multiple levels of the spinal cord enter the superior cervical ganglion through a common nerve and connect with neurons that then innervate distinct peripheral organs. For example, sympathetic neurons innervated by axons from the first thoracic segment controlled pupil dilation, those innervated from the next segment controlled vasoconstriction of the ear, and so on. Langley cut the nerve, awaited regeneration, and asked whether "the fibres of each spinal nerve become connected with only those nerve cells with which they are normally connected, or will they run indiscriminately to such cells as may be on their course…?" The answer was clearly the former: even though axons entered the ganglion together and encountered intermixed targets, they formed functionally appropriate connections.

In a second and more extensive series of experiments, Roger Sperry (Sperry, 1943, 1944, 1963) cut the optic nerves of amphibia (newts, toads, and frogs), then assessed the return of visual function following regeneration. (Central axons regenerate poorly in mammals but well in lower vertebrates.) The lens casts an image of the world on the retina and this image is then processed and transmitted through the optic nerve to form topographic maps in central nuclei. In fact, useful vision was restored, implying that regenerated axons had formed proper connections. Most dramatically, when the eye was rotated, orderly but counterproductive vision was restored: the animal behaved as if it saw the world upside-down and reversed. The clear implication was that retinal axons had reconnected with their original synaptic targets in the brain, not the targets that would now make functional sense. Sperry went on to perform physiological and anatomical experiments that provided definitive support for this view (reviewed in Sperry, 1963).

Langley and Sperry drew similar conclusions. Langley (1895) reasoned that there must be "some special chemical relation between each class of nerve fibre and each class of nerve cell, which induces each fibre to grow towards a cell of its own class and there to form its terminal branches." Sperry (1944) hypothesized that "the ingrowing optic fibers must possess specific properties of some sort by which they are differentially distinguished…[and]… neurons of the optic tectum are also biochemically dissimilar, possessing differential affinities for fibers arising from different retinal quadrants." Moreover, both realized that the recognition was likely to involve interactions along the path that axons follow as they grow toward their targets as well as at the target itself—processes now called axon guidance and target selection, respectively (Langley, 1895; Sperry, 1963).

In retrospect, we see that the power of these experiments came from analyzing regeneration in adults rather than development in embryos. The studies were initially criticized as having limited relevance to how the nervous system wires up as it forms. Yet, during regeneration, confounding factors associated with normal developmental processes, such as precisely timed generation of neurons, orderly arrival of axons, and limitations of spatial access, were eliminated. Sperry's eye rotation experiment even eliminated activity and experience as instructive factors. That is not to say that such factors have no role; indeed

it is now clear that specificity arises from a combination of all of these processes and more (Sanes and Yamagata, 2009; Sanes and Zipursky, 2010). Nonetheless, the work of Langley and Sperry led to a molecular view that remains largely unchallenged: neurons must be chemically specified in ways that guide them to and promote synapse formation with appropriate targets.

Molecules

Following further experiments, Sperry (1963) formalized the chemoaffinity hypothesis, stating that neurons bear "individual identification tags… [with] each axon linking only with certain neurons to which it becomes selectively attached by specific chemical affinities." He believed this individualization could require "millions, and possibly billions, of chemically differentiated neuron types." What sort of molecules might do the trick? Three general possibilities have been suggested.

One is that the differences might be quantitative rather than qualitative with neurons being specified by molecular gradients of adhesive molecules encoding "matching values between the retinal and tectal maps" (Sperry, 1963). Later, Gierer (1983) formalized the model. Based on these ideas, intensive efforts were made to isolate such "gradient molecules"; eventually Bonhoeffer and others showed that complementary gradients of Eph kinases in retina and their ligands, ephrins, in tectum do indeed play critical roles in establishment of the retinotectal and other topographically organized maps (Drescher et al., 1995; Cheng et al., 1995; McLaughlin and O'Leary, 2005). It is less obvious, however, that gradients could endow axons with the ability to distinguish among neuronal types that are physically intermingled rather than spatially arrayed. For example, the specificity required to form microcircuits within the retina or cortex, or connections within invertebrate ganglia, may require qualitatively distinct molecular tags.

A second possibility is that diversity arises from the combined action of many unrelated molecules that act in different ways. Indeed, axons are guided to their targets by a combination of short-range (contact-mediated) and long-range (diffusible) cues that act as both attractants and repellents. Many such guidance molecules and receptors have been identified—ephrins, semaphorins, netrins, plexins, robos, slits, and so on (Dickson, 2002). Most of them turn out to be products of gene families of small or moderate size (up to ~20 for semaphorins). Studies of synaptic specificity suggest that the same mechanisms and, in some cases, the very same molecules act in this process. In the few cases of target recognition that have yielded to analysis, synaptic specificity results from soluble, membrane-bound and matrix-associated proteins of multiple families that act on multiple cell types as both attractants and repellents (Sanes and Yamagata, 2009). This hybrid strategy may seem inelegant, but that does not make it implausible. In fact Jacob (1977) and others have argued that this is how evolution works—as a tinkerer, cobbling together whatever variety of mechanisms are already available as products of prior evolution, not as an engineer, prospectively designing a maximally efficient solution.

Finally, a particularly attractive scenario is that multigene families of adhesion molecules with distinct binding specificities are differentially expressed among neurons of a population and thereby stamp each individual with a distinct identity. This idea was formalized under names such as "area code hypothesis" (Dreyer, 1998). During the 1990s, three families were proposed to play this role: the classical and type II cadherins (~20 genes; Takeichi, 2007), the neurexins and neuroligins (3–4 genes each, but a far larger number of alternatively spliced isoforms; Sudhof, 2008), and the olfactory receptors, a group of ~1000 G protein-coupled receptors expressed by olfactory sensory neurons (Buck and Axel, 1991).

Cadherins, neurexins, and neuroligins have turned out to be critical players in neural development, but to date there is little evidence that they act as determinants of synaptic specificity. The olfactory receptors, in contrast, are clearly required for the precise targeting of olfactory sensory axons to glomerular targets in the olfactory bulb. Each neuron expresses just one of the thousand receptors, and all neurons expressing the same receptor send axons to a single pair of glomeruli in the olfactory bulb. If a receptor is deleted, neurons that would have expressed it innervate the bulb diffusely. When one receptor is genetically replaced by another, the axon is retargeted to an ectopic location, which often corresponds to the proper target of neurons that endogenously expressed that receptor (Mombaerts et al., 1996; Mombaerts, 2006). These "receptor swap" experiments demonstrated an instructive role for olfactory receptors in circuit assembly and led to the speculation that they recognized complementary nonodorant ligands expressed by targets in the bulb. It now seems likely, however, that these receptors act not by interacting with targets directly but rather by differentially modulating levels of intracellular messengers in a ligand-independent fashion; the messengers, in turn, regulate expression of more conventional axon guidance molecules (Sakano, 2010). Thus, olfactory receptors are determinants of specificity, but surprisingly, they act in a rather indirect way.

Are there, then, large families of cell-cell recognition molecules that specify assembly of neural circuits? Over the past few years, two families, the Dscams in insects and the clustered protocadherins (Pcdhs) in vertebrates, have emerged as promising candidates. In both cases, complex genomic loci encode large sets of proteins that are expressed in combinatorial patterns by individual neurons, mediate homophilic binding, and play critical roles in neural development. In the next sections of this Review, we summarize these recent findings. The results lead us to argue that Dscams and Pcdhs are not transsynaptic "chemoaffinity" molecules in the sense that has generally been envisioned. Instead, they contribute to neural specificity in unexpected ways, suggesting a new view of how large families of cell-surface molecules contribute to circuit assembly.

Dscams

Dscam proteins are highly conserved single-pass transmembrane domain proteins of the immunoglobulin (Ig) superfamily (Hattori et al., 2008). Fly Dscam1 was identified through a biochemical interaction of its C-terminal cytoplasmic domain with an adaptor protein, Dock, previously shown to function in growth cones during axon guidance. It comprises 10 Ig domains, 6 fibronectin type III repeats, a single transmembrane domain, and a C-terminal cytoplasmic tail. Like the adaptor, Dscam1 is widely expressed in the developing nervous system and

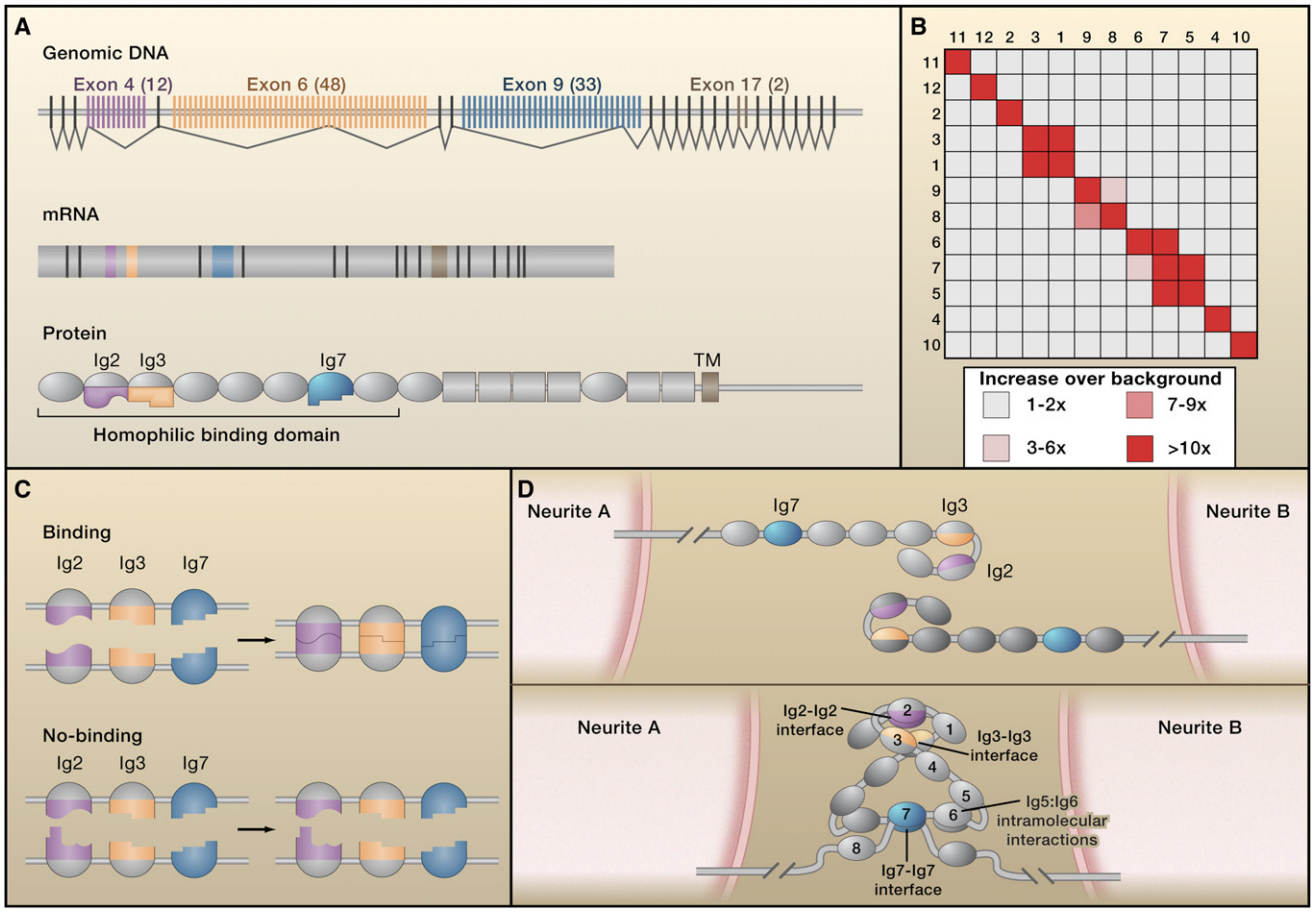

Figure 1. Dscam1 Gene and Proteins

(A) The *Drosophila* Dscam1 gene contains groups of alternative exons that encode 12 different variants for the N-terminal half of Ig2 (purple), 48 different variants for the N-terminal half of Ig3 (orange), and 33 different variants for Ig7 (blue), as well as two different variants for the transmembrane domain (TM) (brown). Splicing leads to the incorporation of one alternative exon from each group, and as such, Dscam1 encodes 19,008 (i.e., 12 × 48 × 33) different ectodomains.

(B) Results of adhesion assays in which Dscams with each of the 12 Ig2 variants were tested for binding to each other. The Ig3 and Ig7 variants were held constant. Each isoform binds to itself but rarely, if at all, to other isoforms. The numbers indicate the alternative Ig2 domain and are arranged as they would be in a dendrogram, such that those closest to each on the grid are closest to each other in sequence. Inset shows binding represented as fold over background (BKGD) (Adapted from Wojtowicz et al., 2007).

(C) Summary of results in (B). Homophilic binding occurs between identical isoforms that match at all three variable Ig domains. Isoform pairs that contain only two matches and differ at the third variable domain bind poorly or not at all to one another. This summarizes the results for Ig2; the properties of the other variable domains are analogous.

(D) Dscam1 monomers have a rigid horseshoe-shaped amino terminus (Ig1–4) and a flexible tail. Dimerization leads to a large conformational change, resulting in a complex of two S-shaped monomers with direct contacts between opposing Ig2, Ig3, and Ig7 variable domains.

essential for guidance of a subclass of axons (Schmucker et al., 2000). Dscam1 thus joined a large group of immunoglobulin superfamily molecules (Shapiro et al., 2007) known to function as receptors for transmembrane and soluble molecules that guide axons to their targets.

Sequence analysis of Dscam1 cDNAs and the genomic locus revealed a feature that set it apart from other neuronal Ig superfamily members, including *Drosophila* Dscam2–4 and the vertebrate Dscams: its primary transcript is subject to massive alternative splicing (Figure 1A). The Dscam1 gene in *Drosophila* and other arthropods contains four blocks of tandemly arranged alternative exons. In *Drosophila*, these encode 12, 48, 33, and 2 variations on Ig2, Ig3, Ig7, and the transmembrane domain, respectively (although the same domains come in alternative flavors in other species, the number of variants and their sequences are highly variable). Any individual mature mRNA contains just one exon from each block. As splicing at each block is independent of the other three, the Dscam1 locus has the potential to encode 19,008 ectodomains (12 × 48 × 33) tethered to the membrane by one of two alternative transmembrane segments.

A first clue to the mechanisms by which Dscam1 functions came from studies of the mushroom body (MB), a central brain structure involved in learning and memory. Each MB contains some 2500 neurons; their axons bifurcate at a common branch point, and the resulting sister branches then segregate to two different pathways (Figure 2A). Removing Dscam1 from all MB neurons led to massive disruption, but removing Dscam1 from

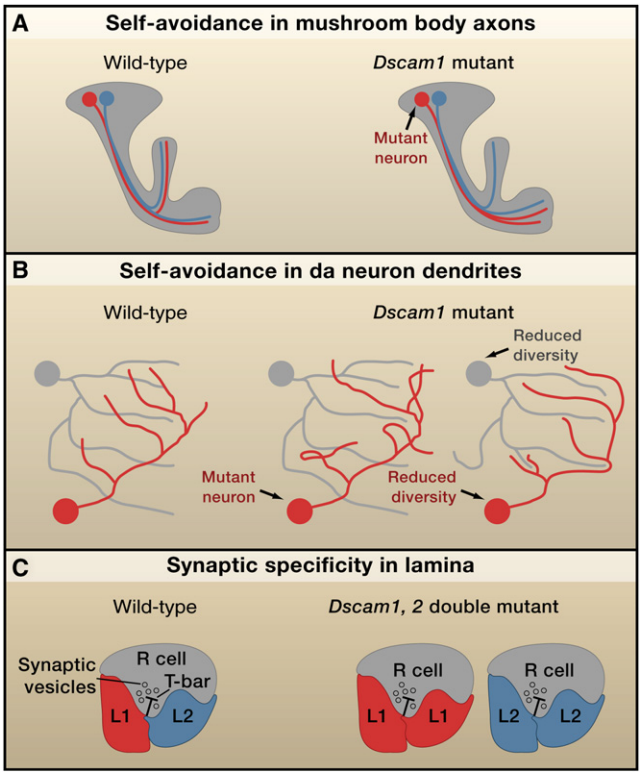

Figure 2. Multiple Roles of Dscam1 and 2 in Neural Development

(A) Dscam1 mediates self-avoidance in axons of mushroom body (MB) neurons. Each of the MB neurons (2 of 2500 are shown) extends a single axon that bifurcates and sends one branch medially and the other dorsally. Each MB neuron expresses a unique combination of isoforms. As a consequence, sister branches recognize each other through Dscam1 matching. This signals repulsion and subsequent segregation of axons to separate pathways. When Dscam1 is removed from a single MB neuron, its branches often fail to segregate.

(B) Dscam1 mediates self-avoidance in dendrites of da sensory neurons. Dendrites of each neuron are splayed out but can cross dendrites of other da neurons. As the dendrites extend on a flat surface, crossing is associated with direct contact between arbors. Deletion of Dscam1 from a single da neuron leads to disordered arbors in which dendrites from the same cell sometimes fasciculate or cross each other. Reducing Dscam1 diversity in all da neurons leads to segregation of their dendrites from each other.

(C) Dscam1 and Dscam2 act redundantly to pattern synapses of photoreceptor (R) axons on L1 and L2 dendrites in the lamina. In each cartridge, R axons form tetrad synapses in which postsynaptic partners always include one L1 and one L2; the other pair comprises combinations of elements from other cell types. They lie above and below the L1/L2 pair (not shown). The T-bar is a presynaptic structural specialization. In the absence of Dscam1 and 2, the repulsion between prospective postsynaptic elements of L1s and between L2s is lost, so some tetrads include two elements from the same L1 or same L2 cells.

single MB neurons in an otherwise wild-type background gave a more interpretable result: the two sister branches of the mutant neuron formed but frequently failed to segregate to the two different pathways (Wang et al., 2002a). This finding, together with biochemical and expression studies described below, led to the notion that homophilic binding of Dscam1 proteins on sister branches from the same cell promotes repulsive interactions between them, thus ensuring that they diverge and grow along separate pathways (Zhan et al., 2004; Wojtowicz et al., 2004). Dscam1 proteins also promote repulsion between dendrites of the same cell (Zhu et al., 2006; Matthews et al., 2007; Hughes et al., 2007; Soba et al., 2007). This process is best characterized in the dendrites of sensory "dendritic arborization" or da neurons (Figure 2B). The da dendrites elaborate highly branched sensory endings in the body wall. As dendrites arborize in a narrow plane, one might expect that dendrites from the same cell would frequently encounter and cross over one another, but such self-crossing seldom occurs. In the absence of Dscam1, however, self-dendrites cross frequently. Thus, as in MB neurons, Dscam1 promotes the repulsion of processes of the same cell. This selective repulsion between dendrites of the same cell promotes uniform coverage of a receptive field while allowing processes of different neurons to share the field.

Recently, Millard et al. (2010) found that Dscam-mediated repulsive interactions among prospective postsynaptic elements also contribute to synaptic specificity (Figure 2C). In vertebrates, typical synapses comprise a presynaptic terminal and a single postsynaptic element. In flies, however, the majority of synapses are multiple contact synapses with a single presynaptic site releasing neurotransmitter onto 2–5 postsynaptic elements. The best characterized of these are so-called tetrad synapses between presynaptic terminals of photoreceptor neurons and postsynaptic elements of lamina neurons (Sanes and Zipursky, 2010). Each photoreceptor axon makes some 50 tetrad synapses, with each tetrad containing two invariant elements, one each from an L1 and an L2 neuron; all 50 tetrads comprise postsynaptic elements from the same two cells. Dscam1 acts in a redundant fashion with its paralog, Dscam2, to control tetrad composition. In the absence of both Dscam1 and Dscam2, the invariant pairing breaks down with many tetrads comprising two L1 or two L2 branches rather than one of each. This phenotype led to a model in which Dscams provide L1 and L2 neurites with the ability to distinguish between self and non-self, thus preventing them from providing two elements to a single tetrad.

Together, these results suggest a common theme to Dscam1 function in multiple aspects of neural circuit assembly: it mediates self-recognition among neurites of a single cell followed by their repulsion from each other. This process was originally observed in leech neurons and termed self-avoidance (Kramer and Kuwada, 1983; Kramer and Stent, 1985). Kramer and colleagues emphasized that self-avoidance was important because it could promote uniform coverage of receptive or projective fields by individual neurons, while allowing multiple neurons to share the same field. More recent studies of Dscams show that self-avoidance can also affect axonal pathfinding and synaptic connectivity. Nonetheless, the phenomenon of self-avoidance was little-studied over the subsequent two decades, perhaps because it was so difficult to envision molecular mechanisms that could allow a neurite to distinguish other neurites of the same cell from neurites of seemingly identical cells—in other words, the problem of distinguishing self from non-self. The chemoaffinity hypothesis provided a framework for seeking molecules that mediate specific intercellular interactions, but there was no corresponding framework for understanding selective interactions among neurites of a single cell.

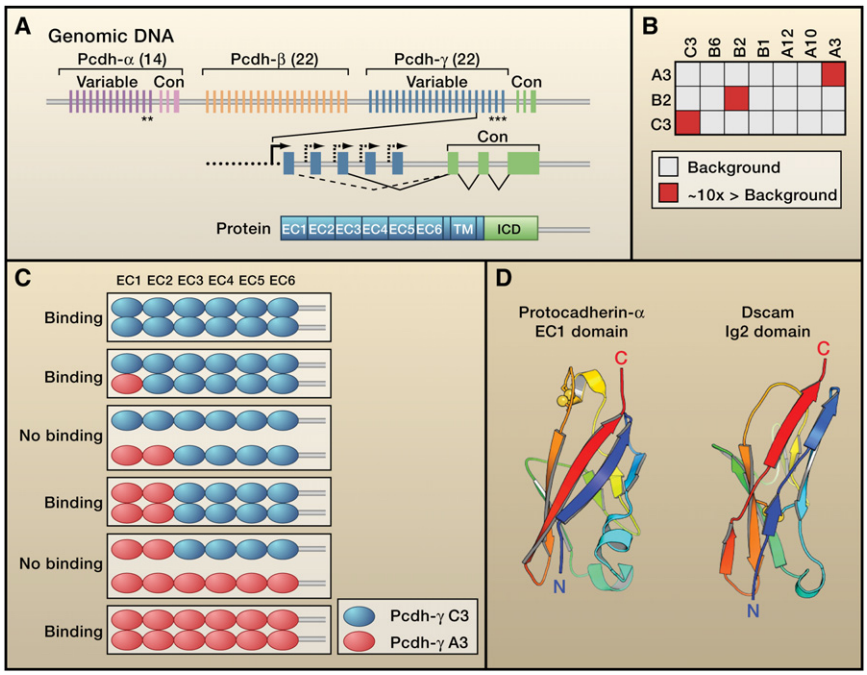

Figure 3. The Protocadherin Gene Cluster and Its Protein Products

(A) The Pcdh gene cluster contains exons that encode 58 extracellular and transmembrane domains—14 in the α group (purple) and 22 each in the β (orange) and γ (blue) groups. Each ectodomain contains 6 cadherin repeats. Ectodcomains are more related to others within a group than to those in other groups with the exception of αC1, αC2, and γC3–5 domains (asterisks), which are more closely related to each other than to neighboring members within their group. Each ectodomain is preceded by a promoter. Alternative splicing joins an α or γ ectodomain/transmembrane exon to three constant exons in the group. β exons encode complete proteins with short intracellular domains.

(B) Results of adhesion assays in which each of seven Pcdh-γs was tested for binding to three isoforms. Each isoform bound preferentially to itself (redrawn from data in Schreiner and Weiner, 2010).

(C) Cadherin domains EC2 and EC3 mediate the specificity of homophilic binding between isoforms (redrawn from data in Schreiner and Weiner, 2010).

(D) Crystal structures of the EC1 domain of Pcdh-α and immunoglobulin domain 7 of Dscam showing the overall similarity of the β sandwich structure of Ig and cadherin repeats.

We will see below that analysis of Dscam1 has provided a way to understand this process.

Protocadherins

There are two Dscam genes in vertebrates (Dscam and DscamL). Early analysis indicates that they promote both class-specific avoidance and transsynaptic target recognition in the restricted subsets of retinal neurons that express them (Fuerst et al., 2008, 2009; Yamagata and Sanes, 2008). However, these are garden-variety genes with few alternatively spliced isoforms, more like fly Dscam2–4 than Dscam1. So, they are unlikely to promote diversity in the way that fly Dscam1 does. However, another set of genes, the clustered protocadherins (Pcdhs; Morishita and Yagi, 2007), show intriguing similarities to fly Dscam1, raising the possibility that they play analogous roles.

In 1998, T. Yagi and colleagues reported identification of a group of eight homologous transmembrane proteins that they called "cadherin-related neuronal receptors" or CNRs (Kohmura et al., 1998). CNRs were fascinating for several reasons. First, their ectodomains placed them squarely within the cadherin superfamily, many other members of which had been implicated in numerous developmental processes (Takeichi, 2007). Second, their expression was largely restricted to the nervous system. Third, immunohistochemical studies showed that they were concentrated at synaptic sites. Finally, sequences of the eight CNRs indicated that they had related ectodomains but identical cytoplasmic domains, suggesting their coexistence in a genomic cluster.

Shortly thereafter, Wu and Maniatis (1999) found that the CNRs are derived from a large genomic locus that encodes a total of >50 genes (58 in mice; Figure 3A) now called clustered protocadherins. (Several other distantly related protocadherins reside at other genomic loci; they are members of the cadherin superfamily generally, but their expression and roles seem quite distinct from those of the clustered protocadherins.) Exons encoding complete extracellular and transmembrane domains are arranged in three groups called Pcdh-α, Pcdh-β, and Pcdh-γ, with 14, 22, and 22 members in the mouse genome, respectively. For the Pcdh-α and -γ clusters, each ectodomain-encoding exon is joined to 3 invariant (constant) exons that encode their common cytoplasmic domain. The Pcdh-β variable exons, which have been less studied to date, appear to encode complete proteins with short cytoplasmic domains; this locus has no constant exons. The cytoplasmic domains of the clustered Pcdhs differ from each other and all lack the canonical catenin-binding domains present in classical cadherins. Like the Pcdh-αs, the Pcdh-β and -γ genes are expressed primarily in the nervous system, and their protein products are concentrated at, but not restricted to, synaptic sites (Wang et al., 2002c; Phillips et al., 2003; Junghans et al., 2008).

Kohmura et al. (1998) and Wu and Maniatis (1999) envisioned several strategies by which Pcdh proteins could be generated from *Pcdh-α* and -γ genes: by genomic rearrangement as occurs in the T cell receptor and immunoglobulin loci, by alternative splicing of a large pre-mRNA, as occurs in *Drosophila* Dscam1, or by alternative use of separate promoters upstream of each first exon. The third alternative is now known to be the correct one (Tasic et al., 2002; Wang et al., 2002b). Each exon is preceded by a promoter and produces a transcript in which the first exon is spliced to the common exons. Pcdh proteins then interact with other products of the cluster to form heteromultimers (Murata et al., 2004; Schreiner and Weiner, 2010). Thus, many Pcdh proteins, like Dscams, are generated from a single genomic locus, though the methods of achieving this diversity are fundamentally different.

Functions of the clustered α and γ Pcdhs have been investigated in targeted mouse mutants. Mice lacking Pcdh-αs are viable and fertile but display subtle neural defects. Perhaps most interesting is a projection error of olfactory sensory neurons. In wild-type animals, axons of olfactory sensory neurons that express the same olfactory receptor converge to innervate a few glomeruli, usually one on each side of the olfactory bulb. In the Pcdh-α mutants, sorting is incomplete, and axons expressing the same receptor end up forming several small supernumerary glomeruli (Hasegawa et al., 2008). Likewise, serotonergic fibers are aberrantly distributed in the brains of Pcdh-α mutants (Katori et al., 2009). Interestingly, the glomerular defects in Pcdh-α mutants show parallels with those observed in the olfactory system of fly Dscam1 mutants (Hummel et al., 2003). These results suggest that Pcdhs play roles in axon guidance or targeting.

Loss of Pcdh-γs, in contrast, leads to devastating neurological defects and neonatal lethality (Wang et al., 2002c). At a cellular level, the most striking phenotype is apoptosis of a substantial fraction of many neuronal subtypes (Wang et al., 2002c; Prasad et al., 2008; Lefebvre et al., 2008; Su et al., 2010). Death occurs during the period of naturally occurring cell death (Prasad et al., 2008; Lefebvre et al., 2008) and appears to be an accentuation of this process. It is observed in many areas and neuronal populations but is not ubiquitous—for example, some neuronal subtypes are spared in retina and spinal cord, and little loss is seen in cerebral cortex, cerebellum, and hippocampus (Wang et al., 2002c; Lefebvre et al., 2008).

The number of synapses is also decreased in Pcdh-γ mutants, but this could be an indirect consequence of decreased neuron number. To test this possibility, the cell death phenotype was largely eliminated by deleting the proapoptotic gene *Bax*. Effects of this manipulation differed between spinal cord and retina: synapse number remained depressed in the former but not in the latter (Weiner et al., 2005; Lefebvre et al., 2008). Moreover, Pcdh-γs appeared to be dispensable for synaptic function and specificity in retina, as electrophysiological recordings indicate that complex computation of visual features can occur in their absence (Lefebvre et al., 2008). Thus, Pcdh-γs may be directly required for synapse formation or maintenance in some but not all regions of the nervous system.

In summary, molecular and genetic studies have revealed that Dscams and Pcdhs are critical for assembly of neural circuits. But do they endow individual neurons with unique identities required to wire up correctly? For this hypothesis to be taken seriously, one would need to demonstrate (1) that individual neurons express distinct sets of Dscams and Pcdhs, (2) that the proteins mediate highly specific intercellular interactions, and (3) that their diversity is required for their function. Recent evidence supports all three of these conditions for Dscams and the first two for Pcdhs.

Combinatorial and Stochastic Expression

Dscam1

The Dscam1 gene encodes 19,008 different ectodomain isoforms. How many are actually expressed, and what cells express them? Sequence analysis of cDNAs prepared from various developmental stages and neuronal subpopulations revealed that all but a single alternative exon were found in mRNA and most were present in multiple populations. More recently, high-throughput sequencing of some 3 million cDNAs from whole animals indicated that more than 17,000 potential combinations of isoforms are indeed expressed (B. Graveley, personal communication).

To gain insight into patterns of isoform expression, Chess and colleagues analyzed cDNAs prepared from purified neuronal subtypes or from single neurons (Neves et al., 2004; Zhan et al., 2004). Little specificity was found in the expression of alternative exons encoding Ig2 and Ig3, although there were cell type-specific biases in the utilization of exons encoding Ig7. Experimental results and an independent statistical analysis generated the estimate that a single neuron expresses 10–50 isoforms. Although it remains unknown whether all mRNAs are translated into proteins, these studies provide strong evidence that Dscam1 isoforms are expressed in a biased stochastic fashion. Thus, as a consequence of alternative splicing and combinatorial expression, Dscam1 appears to endow each *Drosophila* neuron with a unique molecular identity.

Pcdhs

With few isoform-specific antibodies available, expression of individual Pcdh isoforms has been analyzed primarily by in situ hybridization and RT-PCR. Most isoforms are broadly expressed throughout the developing and adult nervous systems, although expression levels vary among isoforms and with age. Expression patterns also vary among isoforms, and some exhibit interesting concentrations in particular laminae or cell types, but the overall impression is one of overlapping rather than mutually exclusive expression at the regional level (Zou et al., 2007; Junghans et al., 2008). Likewise, at the cellular level, double labeling for any two isoforms shows partial overlap (Kohmura et al., 1998; Wang et al., 2002c).

Single-cell RT-PCR analysis of Purkinje cells, chosen because they are large and relatively uniform, provided strong evidence for stochastic, combinatorial expression of Pcdhs in individual cells (Esumi et al., 2005; Kaneko et al., 2006). Each Purkinje neuron expressed 1–3 of the first 12 (that is, 5′) Pcdh-α isoforms and 1–3 of the first 19 Pcdh-γ isoforms. In most cases, expression was monoallelic. There was no obvious relationship between the Pcdh-α and Pcdh-γ isoforms that a Purkinje cell expressed. The 3′ members of each cluster—the final 2 Pcdh-αs and the final 3 Pcdh-γs—exhibited a different pattern. It had already been noted that these 5 isoforms were more closely related by sequence to each other than to neighboring members within their group, and they had been called "C" isoforms (Pcdh-αC1-2 and Pcdh-γC3-5) in recognition of this relationship. All 5 C isoforms were expressed biallelically by Purkinje neurons.

Although limited to Purkinje cells, these data allow estimation of the number of distinct identities that Pcdh-α and -γ expression could confer on neurons. Assuming each cell has the potential to express 1–3 isoforms each of Pcdh-α and -γ, there are some 350,000 possible combinations. Expression of Pcdh-βs considerably increases the number of combinations. These proteins may function in complexes: Pcdh-αs and Pcdh-γs form heteromultimers with no detectable isoform specificity, Pcdh-γs facilitate transport of Pcdh-αs to the cell surface (Murata et al., 2004) and Pcdh-βs associate with α and γ Pcdhs (Han et al., 2010). It is

interesting, though probably coincidental, that if Pcdhs form complexes comprising one of each subfamily, the number of possible combinations (14 × 22 × 22) is similar to that generated by independent inclusion of alternative exons in Dscam1 (12 × 48 × 33).

Dscams and Pcdhs Exhibit Isoform-Specific Homophilic Binding

Dscams

A comprehensive set of binding studies revealed that different Dscam isoforms exhibit an unprecedented range of homophilic adhesive specificities. Wojtowicz et al. (2004, 2007) assayed recombinant proteins containing each of the 12 alternative Ig2s in the context of constant Ig3 and 7 (Figure 1B), each of the 48 Ig3s in the context of constant Ig2 and 7, and each of the 33 Ig7s in the context of constant Ig2 and 3. In nearly all cases, any individual Dscam isoform bound far better to other proteins of the same isoform than to other isoforms, even when the differences between them were small. The very few cases of heterophilic interactions occurred between highly related isoforms. Thus, Dscams show isoform-specific homophilic binding that relies on the matching of all three variable Ig domains (Figure 1C). Based on these studies, it was predicted that the Dscam locus encodes some 18,024 isoforms with isoform-specific homophilic binding (12 × 47 × 32, because one Ig3 variant is not expressed and one Ig7 variant fails to bind).

Two X-ray structures of the Dscam1 Ig domains provided insight into the structural basis for this remarkable binding specificity (Meijers et al., 2007; Sawaya et al., 2008). The eight N-terminal Ig domains form a two-fold symmetric double S-shaped dimer (Figure 1D). The three variable domain interfaces comprise the majority of contacts between the two molecules. Each interface is formed by pairing of a polypeptide strand with the same strand in the opposing molecule in an antiparallel fashion, with binding specificity being determined by the shape and charge complementarity of the interface surfaces. The two sharp turns within the double S-shaped structure, between Ig2 and Ig3 and between Ig5 and Ig6, facilitate the matching of the variable domains in the two opposing molecules. The complementary surfaces of each variable domain fit together like children's blocks.

The structural analysis also provided a way to understand why matching of all three variable domains is required for binding. The Ig2 and Ig3 interfaces are intramolecularly constrained, so a mismatch in either one disrupts matching at the other. Similarly, the four strands at the Ig7 interface are internally constrained, so mismatching between any one prevents the formation of the interface between the others. An intramolecular interface between Ig5 and Ig6 is also crucial for homophilic binding. This interface stabilizes the large conformational change that forms the double S shape on dimerization, thereby bringing the Ig2-Ig3 and Ig7 interfaces together. Thus, the combined interactions at four interfaces (Ig2-Ig2, Ig3-Ig3, Ig7-Ig7, and Ig5-Ig6) lead to all-or-none binding specificity. The conformational change may also initiate the signal transduction process that converts initial homophilic binding into the repulsive response that mediates self-avoidance of sister neurites.

Pcdhs

For many years, attempts to assay adhesive interactions among Pcdh proteins gave equivocal results (Morishita and Yagi, 2007). Very recently, however, Schreiner and Weiner (2010) showed that Pcdh-γs exhibit isoform-specific homophilic binding. They used a novel, quantitative cell adhesion assay to analyze 7 of the 22 different Pcdh-γ isoforms. Each isoform exhibited homophilic binding activity when transfected into cells devoid of endogenous classical cadherins and protocadherins (Figure 3B). Binding specificity was highly reminiscent of the strict isoform-specific homophilic binding exhibited by Dscam1 isoforms.

To explore the molecular basis for this specificity, Schreiner and Weiner asked which of the six Pcdh-γ cadherin domains (EC1–EC6) were required for homophilic binding (Figure 3C). Mutations in EC1 domains disrupted homophilic binding, but swapping EC1 domains between different isoforms did not alter binding specificity. In this respect, protocadherins differ from classical cadherins, in which EC1 is required not only for binding per se but also for isoform specificity (Morishita et al., 2006; Shapiro et al., 2007). Additional domain swaps revealed that both EC2 and EC3 domains contain binding specificity determinants (Figure 3C). Moreover, some chimeras unable to bind either parent were able to bind homophilically; the generation of novel specificities was also a feature of Dscam swaps (Wojtowicz et al., 2007). These findings establish that EC1 is required for binding but not specificity, whereas EC2 and EC3 provide the specificity determinants. Pairing of matched EC2 and EC3 domains from Pcdh molecules on opposing membranes might occur by a strand-swap mechanism, as occurs in EC1 of classical cadherins (Shapiro et al., 2007), or by an antiparallel pairing similar to that found for Ig2 and Ig3 in Dscam1.

Schreiner and Weiner (2010) also extended results of Kaneko et al. (2006) by showing that Pcdh-γs form *cis*-tetramers in an isoform-independent fashion and that this, in turn, expands the binding specificity repertoire. They demonstrated that cells expressing different ratios of Pcdh-γs exhibit selective binding for cells expressing the same ratio. In another interesting experiment, they tested the ability of cells expressing four isoforms to bind to cells transected with the same or different four isoforms. Cells sharing only one or two isoforms bound very poorly whereas cells with three or four shared isoforms showed significant and similar levels of binding. Thus, cells expressing different Pcdh-γ combinations have distinct binding specificities. If Pcdh-αs and -βs contribute to the binding properties of heteromultimeric Pcdh complexes, their combinatorial expression could greatly expand the repertoire of specificities.

Dscam Diversity Is Essential for Patterning Neural Circuit Assembly

As described above, Dscams and Pcdhs are required for numerous aspects of circuit assembly. But is there a special role for their diversity? The question remains unanswered for Pcdhs but has been addressed for Dscam1.

In a first test of whether Dscam1 diversity is required for neural circuit assembly, the genomic region encoding the variable ectodomains was replaced with a cDNA fragment encoding only a single isoform. Marked defects persisted within the peripheral and central nervous systems, including in MB and da neurons,

Table 1. Diversity, Expression, and Roles of Olfactory Receptors, *Drosophila* Dscam1, and Clustered Protocadherins

Feature	Olfactory Receptors	Dscam1	Clustered Pcdhs
# Genes	1000	1	58
Diversity mechanism	Separate genes	Alternative splicing	Promoter choice and multimerization
Expression	1/cell (monoallelic)	10–50/cell	~6/cell[a] (largely monoallelic)
Number of protein products	1000	19,008 ectodomains	12,650 predicted Pcdh-γ tetramers[b]
Ligands	Odorants	Self (homophilic binding) and netrin	Self (homophilic binding of Pcdh-γs); may also have other ligands
Require diversity?	Yes	Yes	Unknown
Mechanism	Modulate second messenger levels intracellularly	Homophilic interactions between processes of a cell leading to repulsion	Unknown
Developmental phenotype	Targeting of olfactory axons to glomeruli in olfactory bulb	Axon and dendrite branching, synaptic specificity	Axon targeting, synapse formation or maintenance, neuronal survival

[a] Assuming an average of two isoforms from each of the α, β, and γ groups, not including αC and γC isoforms.
[b] This estimate is based on the proposal that Pcdh-γs form tetramers (Schreiner and Weiner, 2010) and the assumptions that cells express four Pcdh-γ isoforms and that there is no isoform specificity to multimerization. Diversity could be lower if these assumptions are incorrect or greater in that Pcdh oligomers can contain α and β as well as γ isoforms (see text).

establishing that diversity is, indeed, essential (Hattori et al., 2007).

To determine whether specific Dscam1 isoforms are required, Wang et al. (2004) and Hattori et al. (2007) used a series of deletions removing different sets of exons 4. No defects were seen for either MB or da neurons, indicating that self-avoidance does not rely upon any specific isoforms. Indeed, a single arbitrarily chosen isoform is sufficient for normal patterning of a single da or MB neuron, as long as the surrounding neurons express the wild-type gene and, thus, express different isoforms (Figure 2B). This argues that self-avoidance relies solely on differences between the isoforms expressed on neurons rather than the particulars of their identity.

How much diversity is required? To address this question, Hattori et al. (2009) constructed a series of knock-in mutants through homologous recombination, generating animals carrying 12, 24, 576, 1152, or 4752 isoforms. Both MB and da neurons required between 1152 and 4752 isoforms for normal patterning of axons and dendrites. Although extensive diversity (thousands of isoforms) was not required for a neurite from a single neuron to recognize and be repelled from a sister neurite, it was essential to ensure that neurites did not inappropriately recognize non-self as self. Thus, during neuronal differentiation the biased stochastic expression of some 10–50 isoforms and a large repertoire of isoforms from which to choose ensures that each neuron is sufficiently different from its neighbors. This allows them to distinguish between self and non-self with high fidelity and this, in turn, ensures normal assembly of neural circuits.

Conclusions and Speculations

We have emphasized striking molecular parallels between the Dscams and Pcdhs (Table 1), all of which suggest that they may play similar roles. Both are well suited by pedigree to mediate intercellular interactions: they belong to the two largest and best established families of cell adhesion molecules, the immunoglobulin superfamily for Dscams and the cadherin superfamily for Pcdhs (Shapiro et al., 2007). Both are encoded by complex genomic loci, with remarkable mechanisms to produce many proteins from a single locus. For both, expression is generally stochastic and combinatorial rather than cell type specific, endowing neurons with large numbers of individual identities. Finally, both exhibit isoform-specific homophilic binding by a mechanism involving interactions of multiple Ig (Dscam1) or cadherin (Pcdh) domains.

Perhaps their most striking similarity, though, is their failure—shared with olfactory receptors—to conform to a long-held expectation of how synaptic connectivity is encoded. Sperry (1963) hypothesized that neurons bear "individual identification tags" that encode "specific chemical affinities." But there is no evidence to date that olfactory receptors, Dscams, or Pcdhs act as transsynaptic "locks and keys" to match pre- and postsynaptic partners. Instead, olfactory receptors regulate intracellular messenger levels, Dscam1 mediates self-avoidance, and the most striking role for Pcdhs identified so far is in neuronal survival. Put bluntly, it is hard to imagine that families will be found that are better suited than these to function as chemoaffinity molecules. So, if they don't serve this function, we need to seriously consider the possibility that there is something wrong with the conventional view. We argue that only limited diversity is required for synaptic recognition and that the large-scale diversity that does exist serves other purposes.

How many recognition molecules are required to form appropriate synaptic connections? In fact, in most regions of the developing central nervous system, an ingrowing axon is faced with the task of distinguishing among several to several dozen cell types, not the "millions and perhaps billions" that Sperry (1963) envisioned. The neuron's birth will have placed it out of reach of many of the neuronal types present in the nervous system as a whole. Complex navigational machinery will have guided the growth cone to a restricted target region, narrowing the range of options still further. Within the target, the choice of individual synaptic partners from a class of essentially equivalent neurons may not matter much, and to the extent that it does

matter, it may be regulated by quantitative topographic gradients of a few key molecules (Gierer, 1983). Thus, choices required for synaptic specificity may be mediated in a fashion analogous to those made by growth cones during axon guidance based on the repeated reuse of a limited set of cell recognition and secreted molecules.

If Dscam and Pcdh do not underlie transsynaptic recognition, what is their purpose? Expression patterns provide a clue. For these molecules to function in synaptic matching, the adhesive repertoire of the two partners would need to be precisely matched, requiring exquisite control of splicing or promoter utilization. It is hard to imagine mechanisms capable of such coordination. Indeed, although this type of regulation may occur in some cases, expression appears largely stochastic rather than determinative. Stochasticity is poorly suited to intercellular recognition and synaptic selection but perfectly suited to self-recognition and self-avoidance. The reason is that self-recognition requires each cell to distinguish itself from all of the other cells it encounters (often many thousands), whereas synaptic recognition, as we have argued, may require distinctions among just a few dozen neuronal classes. When diversity is decreased, for example genetically (Hattori et al., 2009), multiple neurons would bear many of the same isoforms, and a neuron would be likely to mistake a neighbor's neurite for its own. The combined features of vast diversity, isoform-specific binding, and stochastic gene expression, which Dscam1 and Pcdhs share, provide a simple and elegant way to provide all neurons with an ability to distinguish between self and non-self. Indeed, the functional experiments reviewed here show that Dscam1 proteins work precisely this way to promote self-avoidance. Self-avoidance, in turn, allows patterning of dendritic arbors and axon branches, thereby promoting uniform coverage of receptor fields and branch segregation. Given their complementary phylogenetic distribution—Dscam diversity occurs in arthropods but not vertebrates, whereas clustered Pcdhs occur only in vertebrates—it is attractive to speculate that Pcdh diversity plays roles in vertebrates similar to those played by Dscam1 in flies. In principle, this idea can be tested genetically in mice, as has been done in flies, by deletion and reintroduction of specific Pcdhs isoforms.

In summary, then, recent studies of vertebrates and invertebrates argue that chemospecificity does exist on the scale envisioned by Sperry but does not play the roles that have generally been envisioned for it. Although chemoaffinity seems likely to underlie synaptic specificity, the number of tags required may be limited. Conversely, cells do carry "identification tags" that enable distinctions "almost at the level of the single neuron" (Sperry, 1963), but these tags act for self-recognition, an issue unanticipated by Sperry and largely ignored by his successors. So in the end, Sperry was right about the need for individual cell identification tags, but we suspect that he would have been surprised by the step in circuit assembly where they are required.

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