The Molecular Basis of Self-Avoidance

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Abstract
Self-avoidance, the tendency of neurites of the same cell to selectively avoid each other, is a property of both vertebrate and invertebrate neurons. In Drosophila, self-avoidance is mediated by a large family of cell recognition molecules of the immunoglobulin superfamily encoded, via alternative splicing, by the Dscam1 locus. Dscam1 promotes self-avoidance in dendrites, axons, and prospective postsynaptic elements. Expression analysis suggests that each neuron expresses a unique combination of isoforms. Identical isoforms on sister neurites exhibit isoform-specific homophilic recognition and elicit repulsion between processes, thereby promoting self-avoidance. Although any isoform can promote self-avoidance, thousands are necessary to ensure that neurites readily discriminate between self and nonself. Recent studies indicate that a large family of cadherins in the mouse, i.e., the clustered protocadherins, functions in an analogous fashion to promote self-avoidance. These studies argue for the evolution of a common molecular strategy for self-avoidance.
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### INTRODUCTION

Important discoveries over the past two decades have transformed the study of neural circuit assembly from the descriptive to the molecular. In the late 1980s and early 1990s, researchers identified key axon guidance molecules, including netrins (Hedgecock et al. 1990, Ishii et al. 1992, Kennedy et al. 1994, Serafini et al. 1994), slits (Brose et al. 1999, Kidd et al. 1999, Li et al. 1999), and semaphorins (Kolodkin et al. 1993, Luo et al. 1993) and their receptors. These act in various combinations to control the directed motility of axons and dendrites in many different developmental contexts in both vertebrates and invertebrates. In the 1990s, investigators also determined that topographic maps, a prominent feature of the vertebrate visual system, form via gradients of Ephrins and Ephs on growth cones and their targets (Cheng et al. 1995, Drescher et al. 1995). In addition, other receptors such as cell adhesion molecules of the cadherin and immunoglobulin (Ig) superfamilies mediating interactions between opposing cell surfaces and integrins acting as receptors for extracellular matrix components are widely expressed on developing neurites (Neugebauer et al. 1988, Tomaselli et al. 1988). Together, these studies led to the view by the late 1990s that neural circuit assembly emerged as a result of a relatively small number of different signals and their receptors, some acting in a graded fashion and in different combinations (Tessier-Lavigne & Goodman 1996). Studies over the past decade have underscored the key
role of this core set of intercellular signaling molecules in regulating circuit assembly in a wide variety of vertebrate and invertebrate systems.

The discovery some 10–15 years ago of two large families of cell surface proteins encoded by the *Drosophila* Down syndrome cell adhesion1 (*Dscam1*) locus and the clustered protocadherin (Pcdh) loci in mammals raised the possibility that these proteins, with diverse extracellular domains and shared cytoplasmic presumptive intracellular signaling domains, could provide diverse recognition specificities to a vast array of different neurites and convert these to a common output (Kohmura et al. 1998, Wu & Maniatis 1999, Schmucker et al. 2000). One obvious and intriguing possibility was that these molecules provided recognition between pre- and postsynaptic neurites. Although a role in synaptic matching remains possible, a series of studies on Dscam1 in flies and recent studies on clustered Pcdhs in the mouse have uncovered a shared molecular strategy by which these families of cell recognition molecules endow neurons with a unique cell surface identity that, in turn, allows neurons to distinguish between self and nonself. This discrimination lies at the heart of a phenomenon called self-avoidance, a process proposed many years ago as crucial for patterning neural circuits but that has received little attention until recently.

The concept of self-avoidance arose roughly 40 years ago from studies of highly organized receptive fields of the medicinal leech, *Hirudo medicinalis*. Mapping of touch receptor axonal receptive fields revealed distinct types of boundaries created between “self” and “nonself” axons. At the outer margins of receptive fields neighboring cells showed overlapping innervation. By contrast, boundaries between stereotyped branches of the same cell were sharp and well-defined, indicating little or no overlap between them (Nicholls & Baylor 1968). Nicholls & Baylor (1968) suggested a mechanism for the spatial arrangement of axons in which “a fiber might repel other branches more strongly if they arise from the same cell than if they come from a homologue, and not at all if they come from a cell with a different modality,” although other mechanisms involving interactions with skin or underlying tissue were not ruled out. Yau (1976) confirmed their findings and proposed that the branches of a cell recognized each other to avoid growing into the same territory.

Kramer & Stent (1985) provided the first experimental support for self-recognition and repulsion between branches of the same cell. They described a large mechanoreceptor neuron that extends three branches, each into one of three contiguous body segments where each then elaborates a complex branching pattern providing uniform coverage of the segments. The branches within each segment did not overlap with one another, nor did they overlap with the processes from the same neuron in the adjacent segment. In animals in which one axon and its terminal arborization were eliminated, neighboring branches from the same neuron grew into the territory that was vacated by the manipulation. This phenomenon was coined “self-avoidance” to indicate the involvement of selective recognition and avoidance between sister branches. Researchers envisioned that self-avoidance was a universal patterning mechanism for axons and dendrites, in vertebrates and invertebrates, and in both the central and peripheral nervous systems (Kramer & Kuwada 1983, Kramer & Stent 1985). Self-avoidance would ensure even spreading of arbors across their territory, and the ability to discriminate between self and nonself would permit coexistence of arbors, ensuring parallel streams of information processing for various types of neurons sharing the same receptive field.

Here we review the role of fly *Dscam1* in regulating self-avoidance. We also discuss recent studies demonstrating that clustered Pcdhs play a similar role in the mouse. These studies suggest that vertebrate and fly neurons have solved the self-recognition problem in fundamentally similar ways via a common molecular principle.
DROSOPHILA Dscam1 PROTEINS REGULATE SELF-AVOIDANCE

A molecular basis for neurite self-avoidance was first uncovered through biochemical and genetic studies of Drosophila Dscam1. As much of this work has been reviewed previously (Zipursky et al. 2006, Millard & Zipursky 2008, Hattori et al. 2009, Schmucker & Chen 2009, Shi & Lee 2012), here we review the key findings in an abbreviated form and focus on more recent studies.

Dscam1 Isoforms Exhibit Isoform-Specific Homophilic Recognition

Dscam1 encodes a family of cell surface receptors, each of which comprises an ectodomain with 10 Ig domains, 6 fibronectin type III repeats, a transmembrane domain, and an intracellular C-terminal tail. Alternative splicing of three large blocks of alternative exons arranged in a cassette-like fashion encodes variable Ig domains giving rise to as many as 19,008 isoforms that differ in one or more of three Ig domains, Ig2, Ig3, and Ig7 (Figure 1) (Schmucker et al. 2000). These diverse ectodomains lie at the center of the mechanism by which neurites of different cells discriminate between one another. In addition to these exons, a pair of alternatively spliced exons encode different transmembrane domains and some adjacent sequences; these differentially control localization of Dscam1 proteins to axons and dendrites (Wang et al. 2004, Shi et al. 2007, Yang et al. 2008). Finally, alternative splicing also generates different variants of the cytoplasmic domain, although how these variants contribute to Dscam1 function is not known (Yu et al. 2009).

The key to Dscam1 function lies in its isoform-specific homophilic binding, as has been demonstrated in vitro via studies ranging from traditional cell aggregation studies to a variety of biochemical assays (Wojtowicz et al. 2004, 2007; Matthews et al. 2007). Binding between isoforms relies on matching of the three variable Ig domains (i.e., Ig2 to Ig2, Ig3 to Ig3, and Ig7 to Ig7) (Figure 2a). Typically, isoforms must match at all three variable domains for binding to occur, although some binding is seen between isoforms in which two domains match and the third is highly related. Isoform-specific binding has been shown for more than 100 isoforms. However, using analytical ultracentrifugation, investigators have assessed the binding affinities for only a few isoforms (Wu et al. 2012; G. Alsen & L. Shapiro, personal communication). The \( K_d \) values fall between 1 and 14 \( \mu M \), a range similar to that observed for other cell adhesion molecules such as classical...
cadherins. Importantly, dimers of isoforms differing in only a single Ig2 domain were not detected (i.e., >500 μM), underscoring the importance of matching at all three Ig domains for binding to occur (Wu et al. 2012) (Figure 2a).

Biochemical (Wojtowicz et al. 2004, 2007; Wu et al. 2007; Sawaya et al. 2008) and structural (Meijers et al. 2007; Sawaya et al. 2008) studies demonstrated that homophilic binding involves pairing of each variable domain with the same variable domain in its binding partner. The structure of a large fragment comprising all three variable domains (Ig1–8), which is sufficient to promote homophilic binding equivalent to the entire ectodomain, revealed that Dscam1 adopts a twofold symmetric S shape (Figure 2a). Two tight turns, one between Ig2 and Ig3 and the other between Ig5 and Ig6, facilitate alignment of each variable domain in an antiparallel fashion; recognition between each cognate variable domain pair relies on a matching via charge and shape complementarity achieved through this antiparallel alignment of the two domains (for the Ig2 interface, see Figure 2b). Extensive binding studies in which the binding specificity of each variant of a variable domain was tested for binding to all other variants of the same domain argued that 18,048 Dscam1 isoforms show isoform-specific homophilic recognition (Wojtowicz et al. 2007).

Dscam1 Isoform Expression in Individual Neurons

The expression of Dscam1 isoforms has been examined in only a few cell types, including mushroom body neurons located in the developing central brain, postmitotic neurons from the eye-antennal imaginal disc, and photoreceptors (R3/R4 and R7 subtypes). Thus, isoform expression analysis represents a major limitation in our current understanding of Dscam1 function. Neves et al. (2004) developed a custom microarray for probing all 93 alternative Dscam1 exons comprising the extracellular domain and examined expression of splice variants in populations of cells isolated by fluorescence activated cell sorting. These studies assessed the distribution of variable domains, but the distribution of different combinations of variable exons giving rise to distinct isoforms could not be determined. Although there was little difference in the distribution of exon 4 and exon 6 variants, some preferential utilization of exon 9 was observed. For instance, using clustering analysis, the researchers determined that the two populations of photoreceptors expressed significantly different exon 9 variants from one another and also from imaginal disc tissue. Different variants of exon 9 were also expressed in single neurons of the same cell type. These studies, together with those of Zhan et al. (2004) in mushroom body neurons, support the view that each neuron expresses between 10 and 50 different isoforms. Chess and colleagues characterized this pattern of Dscam1 as biased stochastic expression (Neves et al. 2004). These studies have led to the view that each neuron expresses a set of Dscam1 isoforms largely different from their neighbors. Additional studies are necessary to assess critically the expression of Dscam1 isoforms, particularly in dendritic arborization (da) neurons (see below), in which our understanding of how Dscam1 regulates self-avoidance is most advanced.

Sorting Out Dscam1 Function In Vivo

Dscam1 function has been studied using knock-out, knock-in, and isoform overexpression approaches in several different cell types in the central and peripheral nervous systems of Drosophila (Schmucker et al. 2000; Wang et al. 2002a; Wojtowicz et al. 2004; Zhan et al. 2004, Zhu et al. 2006; Chen et al. 2006). Much of this work has been reviewed extensively, so we summarize these findings only briefly, and focus primarily on the role of Dscam1 in self-avoidance in da neurons where the most compelling case for its role in self-avoidance can be made. We then consider more recent studies in da neurons, addressing the requirement for homophilic recognition in vivo, the number of isoforms required for neurites to discriminate between self and nonself, and the interplay between self-avoidance and guidance factors. In the final section, we consider the role of Dscam
proteins in regulating synaptic specificity in the visual system.

Da neurons are a group of sensory neurons in the embryo, larva, and adult with highly branched and extensively overlapping dendritic arbors. These neurons detect diverse stimuli and transmit this information from the body wall to the central nervous system. There are four classes of da neurons (classes I–IV) that differ markedly in dendritic complexity and axonal projection pattern (Grueber et al. 2002, 2007). The organization of da neuron dendrites...
is defined by three basic features: (a) Dendrites of different classes of da neurons that share the same receptive field overlap (Figure 3e). (b) Dendrites of the same class of cells typically do not overlap. For instance, the dendrites of class IV da neurons completely cover the body wall but do not overlap with each other (Figure 4a). This phenomenon is typically referred to as tiling. (c) Dendrites from the same cell, or sister dendrites, avoid crossing each other and thus uniformly cover the receptive field (Grueber et al. 2002, 2003; Sweeney et al. 2002; Sugimura et al. 2003) (Figures 3c and 4a). All four classes of da neurons exhibit this self-avoidance property. Tiling and self-avoidance ensure that the body wall is covered completely and nonredundantly by dendrites of each cell class. Coexistence of dendrites of different cell classes ensures that redundant streams of sensory information are received and transmitted from each point on the body wall. Thus, self- versus nonself-discrimination is central to the patterning of da neuron dendritic fields.

Dscam1 Controls Self-Avoidance of Dendritic Arborization Neuron Dendrites

At the single cell level, da neuron dendrites are almost planar in organization, which makes the nearly perfect nonoverlapping pattern of self-dendrites very apparent (Grueber et al. 2002, Sweeney et al. 2002) and thus favorable for sorting out the molecular control of self-avoidance. The relative simplicity and planar arrangement of da neurons facilitate quantification of self-avoidance by measuring the incidence of self-dendrite crossing or fasciculation. Furthermore, researchers can quantify discrimination between self and nonself in different mutant backgrounds by assessing crossover between the processes of different da neurons (see below) (Figure 3a–d). Loss of Dscam1 in single da neurons (that are surrounded by normal neurons in genetically mosaic animals) causes severe cell-autonomous defects in self-avoidance (Hughes et al. 2007, Matthews et al. 2007, Soba et al. 2007). This phenotype was seen in all da sensory neurons examined, indicating a shared requirement for Dscam1 in self-recognition and repulsion. As a consequence of the self-avoidance phenotypes, coverage of territories was incomplete with unusually large gaps between branches (Figure 3b).

The analysis of Dscam1’s role in self-avoidance in da neurons has led to four key conclusions. First, no particular Dscam1 isoform is required for self-avoidance, as self-avoidance phenotypes were not observed in a series of alleles analyzed in which, in aggregate,
Dendritic arborization neurons

Starburst amacrine cells

Figure 3

Dscam1 and Pcdh-γ genes regulate self-avoidance. (a, b) Dscam1 is required for self-avoidance (reproduced with permission from Matthews et al. 2007). The branches of a single wild-type type III neuron in the body wall of a Drosophila larva seldom cross one another. By contrast, processes of Dscam1 mutant cells cross and often fasciculate (arrows) with each other. (c, d) Ectopic expression prevents coexistence. (i) In wild type, cells of different classes of da neurons frequently share the same receptive field. Thus, dendrites of different cells cross one another: (magenta) type I dendritic arborization (da) neuron, (green) type III neuron. (d) Ectopic expression of the same isoform of Dscam1 in type I and type III neurons promotes repulsion between their dendrites. As a consequence, these two cells do not cover the same receptive field. (e, f) Pcdh-γ is required for self-avoidance. (e) Wild-type starburst amacrine cell (SAC) processes show little overlap, particularly in the regions proximal to the cell body. (f) SACs lacking all 22 Pcdh-γ isoforms are highly fasciculated. These images were kindly provided by J. Lefebvre and J.R. Sanes. (g, h) The relative overlap between the processes of SACs in cells ectopically expressing the same isoform is reduced compared with wild type (reproduced with permission from Lefebvre et al. 2012). The relative overlap is shown between real images and between images in which one of the two cells is flipped (left-hand image; blue bar in panel b). The overlap is greater in the real image in the wild-type than in the rotated images, arguing for a positive interaction between processes of the different SACs. A reduction in overlap is indicated between isoforms if both cells ectopically express the same Pcdh-γ isoform, in this case cA1 (right-hand image; red bar in panel b). An image between mutant cells lacking Pcdh-γ (Pcdh-γ^{rko/rko}) is not shown.

all 12 versions of exon 4, and thus all possible isoforms, were removed. Second, Dscam1 diversity is not required in individual neurons for self-avoidance; single arbitrarily chosen isoforms rescue the Dscam1 null self-avoidance phenotype. Third, Dscam1 diversity is essential for discriminating between self- and nonself-dendrites, because overexpression of single isoforms in neurons whose dendrites overlap in wild-type animals led to their segregation into nonoverlapping territories (Hughes et al. 2007, Matthews et al. 2007, Soba et al. 2007) (Figure 3d). Fourth, expression of single Dscam1 molecules lacking most of their cytoplasmic tail prevented ectopic branch segregation and instead led to apparently stable adhesion between dendrites. How the cytoplasmic domain promotes repulsive interactions remains unknown. In sum, isoform identity between branches of the same neuron leads to recognition via the extracellular region and repulsion mediated by the intracellular tail.
As the Dscam1 isoforms expressed in different da neurons are likely to be different, dendrites of different da neurons do not inappropriately recognize nonself as self. Thus, Dscam1 proteins are required for self-avoidance and provide the molecular code by which neurites discriminate between self-dendrites and those of neighboring cells.

**Homophilic Recognition Provides the Molecular Basis for Self-Avoidance**

To test whether homophilic binding of Dscam1 isoforms is required for self-avoidance, Wu and coworkers generated homophilic binding defective mutants (Wu et al. 2012). In this study, pairs of mutant isoforms were engineered to lose homophilic binding and to simultaneously acquire heterophilic binding specificity to each other (Figure 2). Efforts to alter specificity focused on modifying alternative versions of exon 4, which encode variable Ig2 domains. By comparing 89 Ig2 interface segments of Dscam and Dscam1 proteins from 39 species, researchers generated pairs of chimeric Ig2 domains with desired specificity in vitro and introduced them into flies to test for their ability to support self-avoidance (Wu et al. 2012) (Figure 2).

The ability of single chimeric isoforms (Dscam1 single chimeras) knocked into the endogenous Dscam1 locus to support self-avoidance was compared to single isoforms that retained homophilic binding properties (Dscam1 single). Dscam1 single isoforms shared the same Ig3 and Ig7 domains with mutants, but they encoded wild-type Ig2 domains. Whereas Dscam1 single isoforms were sufficient for self-avoidance in different classes of da neurons, Dscam1 single chimeras isoforms showed reduced ability to support self-avoidance. Dscam1 single chimeras isoforms were also compromised in their ability to induce ectopic repulsion between different da neuron dendrites when overexpressed. Nevertheless, nonmatching isoforms still retained some activity both when expressed from the endogenous locus and in overexpression experiments, arguing that weak binding between isoforms, matching at only two variable domains, is sufficient to induce low levels of repulsion (Wu et al. 2012). Weak binding may arise from avidity between oligomerized proteins on the cell surface (J.J. Flanagan & S.L. Zipursky, unpublished results), as these proteins showed no detectable binding affinity using analytical ultracentrifugation (i.e., a measure of trans-dimerization). In contrast to the weak ability of Dscam single chimeras isoforms to promote repulsion, coexpression of two complementary chimeric isoforms in da neurons with overlapping dendrites in wild type promoted ectopic repulsion between them, resulting in the establishment of nonoverlapping dendritic fields (Wu et al. 2012). Homophilic binding is also important for axonal branching in mushroom body neurons. Indeed, coexpression of complementary chimeras in single Dscam null mutant mushroom body neurons rescued self-avoidance defects. Together, these results provide strong evidence that binding between Dscam1 isoforms on opposing surfaces of neurites of the same cell is required for self-avoidance (Wu et al. 2012).

**Diversity at the Dscam1 Locus Is Essential for Discrimination Between Self- and Nonself-Neurites**

Although analysis of mutants in which the ectodomain diversity was reduced to a single isoform through a knock-in replacement strategy clearly showed that Dscam1 diversity was required for discrimination between self and non-self (Hattori et al. 2007), researchers did not know how many isoforms were necessary to ensure that neurites do not inappropriately recognize and avoid nonself-neurites. To address this question, Hattori et al. (2009) took a genomic replacement strategy in which alternative exons were replaced with cDNAs encoding exons 7–11 (i.e., to fix exon 9 and limit the number of potential isoforms to 576) or exons 5–11 (i.e., to fix exons 6 and 9 and limit the number of potential isoforms to 12). By combining two different Dscam1576 isoform alleles, the number of isoforms was fixed.
at 1152; likewise, use of two different Dscam\textsuperscript{12}-isoform alleles fixed maximum diversity at 24 isoforms. In addition, two deletion alleles that removed blocks of 3 and 9 alternative exon 4s were used to limit the number of isoforms to 14,256 and 4,752, respectively. Thus, these alleles together allowed testing discrimination between self versus nonself in animals carrying no isoforms (null) and those carrying 12, 24, 576, 1,152, 4,752, and 14,256 isoforms.

Self-recognition was normal with all knock-ins (i.e., from 12 or more isoforms). By contrast, inappropriate recognition of nonself as self was seen in knock-ins up to 1,152 isoforms; da neurons discriminated between self and nonself with 4,752 isoforms. Thus, thousands of Dscam1 isoforms are required for discrimination between self- and nonself-dendrites. Similar requirements for thousands of isoforms were observed in other neurons. For example, separation of mushroom body lobes, taken as a measure of self-avoidance between sister axon branches segregating to form the two lobes, depends on Dscam1 and is defective when the number of potential isoforms falls to 1,152 (Hattori et al. 2009). Mathematical modeling based on the number of isoforms expressed in neurons and an estimate of the number of shared isoforms allowed (i.e., that do not activate inappropriate repulsion) supports

\textit{Drosophila} dendritic arborization (da) neurons: self-avoidance, tiling

Mammalian starburst amacrine cells: self-avoidance, co-existence
the conclusion that thousands of isoforms at a minimum are required to produce on the order of tens of distinct neuronal identities via a stochastic mechanism (Hattori et al. 2009, Forbes et al. 2011).

**INTEGRATING SELF-AVOIDANCE WITH OTHER DENDRITIC PATTERNING PROCESSES**

**Self-Avoidance and Netrins**

Self-recognition and repulsion occur as dendrites are responding to other extracellular cues that determine their targeting and position; thus these various signals must be integrated by developing dendritic branches. Indeed, studies on da neurons have identified interplay between Dscam1-mediated repulsion and other guidance signals. In Dscam1 null mutant clones, not only did dendritic processes form clumps, but clumping also often occurred at stereotyped positions (Matthews et al. 2007). These clumps corresponded in position to Netrin-B expressing cells that promote specific dendritic targeting of da neurons (Matthews & Grueber 2011). In the absence of either Netrin-B or the netrin receptor Frazzled, dendrites fail to target their normal territory. By contrast, in the absence of Dscam1, dendrites show an opposite “hypertargeting” to the source of Netrin-B. Removal of both Frazzled and Dscam1 signaling causes strong reductions in dendritic fields, and sister dendrites become highly tangled. Thus, integration of Netrin/Frazzled and Dscam1 systems promotes spreading of dendrites from the cell body to more distant targets and prevents sister dendrites from targeting in unison to the source of guidance cues. In this manner, dendrite self-avoidance and guidance cues act together to promote territory coverage. However, the mechanism by which Dscam1-dependent repulsion is integrated with

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**Figure 4**

Organization of dendritic arborization (da) neurons and starburst amacrine cells (SACs), contrasting self-avoidance, tiling, and coexistence. (a, c) Both da neurons and SACs elaborate highly branched dendrites that self-avoid within restricted layers. For da neurons, mechanisms that constrain dendrites to a common surface facilitate self-avoidance. To our knowledge, the detailed surface interactions between developing processes of SACs within a layer have not been described. (a) Drosophila da neurons show both self-avoidance and tiling. The schematic shows a field of class IV da neurons. The processes of these cells do not overlap with processes of the same cell (self-avoidance) or with process of da neurons of the same class (tiling). They do overlap with the processes of different classes of da neurons (not shown). (b) Da neurons arborize dendrites in a narrow plane sandwiched between muscle (green) and epidermis (gray). This planar organization promotes self-avoidance. Complex class IV arbors (red) and a single overlapping class I arbor (blue) are represented. Electron microscopic analysis shows that dendrites in cross section (shaded red) either reside on the basal surface of the epidermis (shaded yellowish-brown in the two photo details) in contact with the extracellular matrix (ECM) (shaded blue in the two photo details) or are enclosed within invaginations of the epidermal membrane. In wild type, tight association of dendrites with the ECM facilitates contact between neurites, thereby promoting repulsion. Envelopment within the epidermal membrane would physically isolate processes and thus antagonize direct contact between them, leading to crossing in a different three-dimensional plane (see text). (c) Mammalian SACs elaborate dendrites that show self-avoidance (visible in black neuron) but that coexist, or overlap, with many neighboring SACs (red). (d) Organization of mammalian retina. Rod and cone photoreceptors reside in the outer nuclear layer (ONL) and extend into the outer plexiform layer (OPL) where they synapse with rod and cone bipolar cells. Cell bodies of bipolar cells, horizontal cells (not shown), tyrosine-hydroxylase (TH)-immunoreactive cells (orange), and SACs (red) reside in the inner nuclear layer (INL). Bipolar and amacrine cells project to different layers of the inner plexiform layer (IPL). SACs reside both in the INL and in the ganglion cell layer (GCL), and they send processes to distinct narrow layers within the IPL, where they overlap extensively with neighboring SAC dendrites. Mammalian DSCAM and DSCAML1 regulate interactions between dendrites of many of these cell types. Pdhr-1 proteins regulate interactions between self-processes of SACs. Drawing in panel d modified with permission from Wassle (2004).
other guidance cues that give dendrites and axons their proper shape and trajectory remains unknown. Counterbalancing roles for Dscam1 could be particularly important in complex neuropils where dendrites respond to a multitude of extracellular cues during targeting and could conceivably help to explain clumping and fasciculation phenotypes seen in several different contexts in Dscam1 mutant brains.

Self-Avoidance and Integrins

An integrin-dependent pathway that constrains da dendrites to a common surface plays a key role in self-avoidance (Han et al. 2012, Kim et al. 2012). Without such a constraint, for instance, in a three-dimensional field, arbors might simply inch past one another. Integrin receptors, which mediate interactions between cell surfaces and the extracellular matrix, normally restrict da dendrites to a single plane (Han et al. 2012, Kim et al. 2012). In the absence of neuronal integrins, some arbors become enclosed within segments of the overlying epidermis and dendrites show excessive overlap (see Figure 4b). By contrast, in response to co-overexpression of α- and β-integrin subunits, more dendrites attach to the extracellular matrix, thus decreasing enclosure. Other components of this two-dimensional restriction pathway include the Tricornered kinase and Furry (Emoto et al. 2004) and components of the TOR complex 2 (Koike-Kumagai et al. 2009, Han et al. 2012). The enclosure of dendrites prevents Dscam1-dependent repulsion, because arbors do not come into contact and, as a consequence, are unable to self-repel. These mechanisms may also function within the neuropil. Here, processes may be constrained to grow along the surface of other cells, such as glia, thereby enforcing interaction between neurites of the same cell.

Self-Avoidance and Synapses

Using electron micrographic studies of developing synapses in the fly visual system, investigators determined that repulsive interactions between prospective postsynaptic elements of the same cell regulate the correct assembly of synapses between photoreceptor neurons and their postsynaptic targets. Photoreceptor cells in the fly retina form multiple contact synapses, or tetrads, with four distinct elements, lamina interneurons L1 and L2, and two other elements (either from a glial cell, a lamina L3, or a lamina amacrine cell) (Meinertzhagen & O’Neil 1991) (Figure 5a,b). Each lamina microcircuit contains only one L1 and one L2 cell. Each cell forms many small dendritic processes, which contribute postsynaptic elements to multiple contact synapses on both the same and six different photoreceptor cell axons (Figure 5b). Pairing ensures that L1 and L2 receive matched inputs from photoreceptor neurons. Such pairing appears to arise from synaptic exclusion and refinement of intermingled pairs of L1/L1 and L2/L2 seen during synapse assembly (Frohlich & Meinertzhagen 1983) (Figure 5b,c), raising the possibility of a self-avoidance mechanism at work to ensure proper tetrad pairing.

Tetrad composition is normal in Dscam1 mutants, and Dscam2 mutant retinas exhibit a small fraction of tetrads with two L2 elements (Millard et al. 2010). In the absence of both Dscam1 and Dscam2, however, tetrad composition is randomized, indicating that Dscam1 and Dscam2 act redundantly to exclude inappropriate synaptic pairing at tetrads (Millard et al. 2010). This phenotype may arise through a defect in self-avoidance, such that during normal development L1-L1 and L2-L2 pairs are eliminated by Dscam1/Dscam2-dependent repulsion leaving L1-L2 pairs at mature tetrad synapses (Figure 5d). As multiple contact synapses are seen throughout the fly visual system, and indeed elsewhere within the fly central nervous system, Dscam-mediated self-avoidance likely contributes in this way to the precise assembly of synaptic connectivity.

Summary

On the basis of classical work largely conducted in invertebrates, researchers proposed that self-avoidance plays a widespread role
in patterning neural circuits. Nevertheless, the phenomenon received little attention, perhaps in large part owing to the difficulty of studying the development of neuronal arbors, particularly in the central nervous system, at the level of single cells. Studies in *Drosophila* over the past decade have not only underscored the importance of self-avoidance in the developing nervous system but also uncovered an underlying molecular strategy regulating this process. In this system, neurons acquire a unique cell surface identity through the expression of different combinations of a vast array of homophilic cell recognition molecules. This enables each neuron to distinguish its own neurites from the neurites of other neurons that it encounters. Binding between identical isoforms signals self and promotes repulsion of processes away from one another.

Importantly, Dscam1 endows each neuron, whether of the same or a different cell type, with a unique identity and thus facilitates neuronal individualization. One consequence of the vast number of different identities is coexistence, either among dendrites of different cell types that must cover overlapping receptive territories or in contexts in which neurites of a single cell must distinguish between sister neurites and neurites of other cells of the same cell type. This latter scenario arises in mushroom body axons in the fly central brain (Wang et al. 2002a, Zhan et al. 2004, Hattori et al. 2009) and, as we discuss below, during dendritic patterning of starburst amacrine cells (SACs) in the vertebrate retina.

**DSCAM Regulates Interactions Between Dendrites of the Same Cell Classes in the Mammalian Retina**

The first clue that DSCAM proteins regulate interactions between processes of the same cell in mammals came from studies on retinal amacrine cells. These cells arborize within a layered neuropil called the IPL where they make synapses with both bipolar neuron axons and the dendrites of retinal ganglion cells (RGCs) (Figure 4d). DSCAM is selectively expressed in two of approximately two dozen classes of amacrine cells whose cell bodies form evenly spaced mosaics across the retina (Fuerst et al. 2008): The tyrosine-hydroxylase-positive and bNOS-expressing amacrine cells arborize in the S1 and S3 layers of the IPL, respectively. Loss of DSCAM leads to fasciculation of tyrosine-hydroxylase and bNOS amacrine cell dendrites and abnormal cell body spacing. In contrast to the selective expression of DSCAM in segregated amacrine cell populations, DSCAM is expressed in the dendrites of virtually all RGCs that also extend into the IPL (Fuerst et al. 2009). Removal of DSCAM results in the fasciculation of dendrites with sister dendrites as well as selective fasciculation with the dendrites of cells of the same class of RGCs. These
Synaptic selection

Competition, exclusion, retraction

Synapse maturation

Self-avoidance

Matching Dscams (1 and 2) Repulsion

Nonmatching Dscams Synapse formation

Matching Dscams (1 and 2) Repulsion
findings led to the conclusion that DSCAM may function primarily to mask cell-type-specific adhesive interactions between dendrites of the same cell class, rather than to actively promote repulsion between them. In this way, DSCAM acts to negate cell-type-specific interactions that are promoted by other, as-yet unidentified, recognition molecules. Such a mechanism would not, however, allow dendrites of one cell to distinguish between self-dendrites and dendrites belonging to other cells of the same cell or different cell types. Thus, although DSCAM mediates self-avoidance for the amacrine cell subclasses expressing it and all RGC dendrites, it does not allow processes of one cell to discriminate between self-processes and the processes of other cells.

The expression of DSCAML1, versus DSCAM, is far more restricted within the mouse retina. It is expressed in cells in the rod circuit, including rod, rod bipolar cells (RBCs), and AII amacrine cells, but it does not appear to be expressed in RGCs (Fuerst et al. 2009). Like DSCAM, loss of DSCAML1 results in cell clustering and neurite fasciculation, a function consistent with self-avoidance. For instance, the dendrites of RBCs appear to be highly fasciculated, and the AII amacrine cells form clumps (Fuerst et al. 2009). Though largely normal synaptic structures were observed between cells in the rod circuit that lacked DSCAML1, some abnormalities were also observed. For instance, there was a marked increase in synaptic vesicles in RBC/AII/A17 synapses suggestive of a defect in exocytosis. In addition, there was a nearly fourfold decrease in synapses between rods and RBCs, which may be a consequence of the ~40% decrease in average length of RBC dendrites, their fasciculation, or both. These defects in RBC dendrite structure and organization may prevent normal extension of RBC dendrites to their rod synaptic targets. Thus, DSCAML1 also appears to regulate self-avoidance. As with DSCAM, DSCAML1 defects are also associated with an increase in cell number. Although experiments on DSCAM support the view that the defects in the organization of neurites are independent

Figure 5

Dscam1 and Dscam2 play redundant roles in regulating the composition of postsynaptic elements at multiple contact synapses. (a) The R1–R6 photoreceptor neurons in the Drosophila retina form synapses with neurons in the lamina within microcircuits called cartridges. The dendrites of target neurons, as is typical of arthropods, extend from the proximal region of the axon. There is only a single cell of each L1–L5 neuron in a cartridge. (b) R1–R6 axons form tetrad synapses. Each R cell axon (gray) forms ~40 presynaptic release sites adjacent to four postsynaptic elements, including an invariant pair from L1 and L2 and a variable pair, in the example shown here an L3 neuron and an amacrine cell (amacrine cells form synapses in multiple cartridges) (not shown). Note each L1 and L2 elaborates many dendrites, yet there are no L1 or L2 pairs. The invariant pairing of L1 and L2 is thought to be important for motion detection. (Inset) A transmission electron microscopy section through a tetrad synapse shows the T-bar (arrow), thus indicating the presynaptic release site or active zone, and the invariant L1 and L2 postsynaptic elements. (c) Model for the formation of invariant pairs of L1 and L2 postsynaptic elements based on studies by Meinertzhagen et al. (2000). The multiple L1 (green) and L2 (blue) dendrites are each from the same L1 and L2, respectively. Pink circle indicates the prospective presynaptic sites. At some sites, two L1 or two L2 processes pair transiently. These are resolved through interactions between processes leading to the establishment of mature synapses (red dot indicates mature presynaptic site) resulting in the invariant pairing of L1 and L2. Black and red arrows indicate retraction and extension, respectively, of postsynaptic elements to establish L1/L2 pairing. The other postsynaptic elements in the tetrad are not shown. (d) Model of Dscam1 and Dscam2 function in regulating tetrad assembly. Dscam1 and Dscam2 are required in a redundant fashion to prevent inappropriate pairing through self-avoidance. Orange and yellow bars represent different Dscam1 isoforms expressed on the surface of L1 and L2. Light blue and purple bars indicate different Dscam2 isoforms (either the A or B isoform) proposed to be expressed on L1 and L2. Because Dscam1 and Dscam2 homophilic binding activates repulsion, only the L1/L2 pairing is permitted. Drawings and inset image reproduced with permission from Millard et al. (2010).
of the cell-death phenotypes (Keeley et al. 2012), whether the morphological phenotypes observed in DSCAML1 retinas are an indirect consequence of this increase has not been addressed.

In summary, Burgess and coworkers have proposed that the mammalian DSCAMs promote an anti-adhesive function, preventing the adhesion between processes of cells of the same type rather than actively promoting repulsion between them (Fuerst & Burgess 2009, Garrett et al. 2012b). Although it remains unclear what a general antiadhesive function might look like at a mechanistic level, in the absence of diversity, mammalian DSCAMs would not provide cells with the ability to distinguish between their own processes and the processes of all other cells, including processes from cells of the same type. As we describe in the next section, clustered Pcdhs provide SACs with precisely this function, thereby utilizing a strategy remarkably similar to Drosophila Dscam1, albeit in the guise of a different protein superfamily.

**PCDH-γS REGULATE SELF-AVOIDANCE**

**Diversity of Pcdh-γs Provides a Basis for the Identity of Vertebrate Neurons**

Just prior to the discovery of the diversity in the Drosophila Dscam1 locus, two studies in vertebrates led to the finding of a large cluster of closely related putative cell recognition molecules of the cadherin superfamily. In 1998, Yagi and colleagues reported the discovery of eight structurally related transmembrane proteins of the cadherin superfamily termed cadherin-related neuronal receptors (Kohmura et al. 1998). These proteins are expressed broadly in the developing nervous system and are localized to synapses. Wu & Maniatis (1999) identified the genomic loci encoding these proteins and demonstrated that these were tightly linked and organized within three distinct clusters. These are now referred to as the clustered Pcdhs with each cluster referred to as Pcdh-α (including the cadherin-related neuronal receptors identified by Yagi and colleagues) and the related Pcdh-β and Pcdh-γ proteins (Figure 6). The number of Pcdh isoforms varies between different vertebrate species, but in aggregate, there are typically on the order of 50 isoforms. In the mouse, for instance, there are 14 Pcdh-α, 22 Pcdh-β, and 22 Pcdh-γ isoforms. Studies of clustered Pcdhs indicate diverse roles in the nervous system, including promotion of neuronal survival (Wang et al. 2002c, Lefebvre et al. 2008, Prasad et al. 2008), synaptic development (Weiner et al. 2005), axon target recognition (Prasad & Weiner 2012), and dendritic arborization (Garrett et al. 2012a).

Clustered Pcdhs have been found only in vertebrates; conversely, vertebrate DSCAMs lack the diversity of their arthropod counterparts. Such findings raised the intriguing possibility that these protein families could play analogous roles in the developing nervous system. Although both clustered Pcdhs and Dscam1 genes generate families of proteins with diverse ectodomains joined to a common cytoplasmic domain, the organization and mode of generating clustered Pcdh and fly Dscam1 diversity are markedly different. Pcdh diversity is largely generated by alternative promoter choice, as opposed to alternative splicing (Tasic et al. 2002, Wang et al. 2002b). Alternative ectodomains are encoded by single large exons arranged in a head to tail fashion (see Figure 6), and each exon is preceded by a transcription start site. For the Pcdh-α and Pcdh-γ clusters, each ectodomain-encoding exon is spliced to a common cytoplasmic tail found within the 3’ end of the cluster. By contrast, each Pcdh-β is encoded in its entirety by a single exon. In addition, the Pcdh-α and Pcdh-γ clusters contain five exons that are highly related to one another; these are referred to as C1 and C2 within the α cluster and as C3–C5 within the γ cluster. Molecular studies argue that variable Pcdh-α and Pcdh-γ are expressed in a largely stochastic fashion (Kaneko et al. 2006). Single-cell polymerase chain reaction analyses were
Figure 6
Alternative transcription initiation generates multiple isoforms of Pcdh-γ. Three clusters of protocadherin (Pcdh) genes are tightly linked to one another. Fourteen Pcdh-α and 22 Pcdh-γ alternative exons encode different ectodomains. These each comprise six extracellular cadherin (EC1–EC6) repeats and a single transmembrane (TM) domain. These variable ectodomain-encoding exons are spliced to a common C-terminal tail [intracellular domain (ICD)]. Each Pcdh-β exon encodes an entire protein isoform tethered to the membrane. Each exon is preceded by a transcription initiation site. Like alternative isoforms of Dscam1, different Pcdhγ ectodomains exhibit isoform-specific homophilic binding. Pcdhγ proteins form tetramers (not shown) that exhibit preferential binding for tetramers of the same composition. Abbreviation: Con, conserved.
undertaken to study Pcdh-α and Pcdh-γ expression in Purkinje cells [Pcdh-β expression was also examined, which was noted as unpublished results discussed in Yagi (2012)]. Each Purkinje cell expresses all C1–C5 isoforms and different combinations of Pcdh-αs (~2 of 12), Pcdh-βs (~4 of 22) (Yagi 2012), and Pcdh-γs (~4 of 19). Thus, a vast number of different combinations of Pcdhs is possible, and as with Dscam1, this would have the potential to endow neurons with a unique cell surface identity.

**Pcdh-γ Isoforms Exhibit Isoform-Specific Homophilic Recognition**

Although the clustered Pcdhs were discovered in the late 1990s, compelling evidence for discrete binding specificities of different isoforms was not uncovered until 2010. By devising an elegant and quantitative assay and a suitable cell line for protein expression, Schreiner & Weiner (2010) convincingly demonstrated that Pcdhs promote isoform-specific homophilic recognition. In their assay, the activity of seven Pcdh-γs was assessed. Of critical importance, of the many cell lines tested in various Pcdh-γ transfection experiments, only the human leukemia cell line K562 supported Pcdh-γ-dependent cell aggregation. To compare homophilic and heterophilic binding, the authors further assessed interactions between two cell populations, one expressing an isoform tagged with a hemagglutinin (HA) epitope and the other expressing either the same or a different isoform along with the enzyme β-galactosidase. These cells were mixed and adsorbed to an anti-HA antibody; bound β-galactosidase enzymatic activity was then measured.

The studies of Schreiner & Weiner (2010) led to several important findings. First, each of the seven Pcdh-γ isoforms tested exhibited isoform-specific homophilic recognition. Second, the Pcdh-γ isoforms oligomerize and appear to form tetramers preferentially. Third, Pcdh-γs form hetero-oligomers in cells co-transfected with different isoforms, and cells expressing the same combination of isoforms preferentially bind to themselves rather than to cells expressing different combinations (even if some isoforms are shared between the cells). Studies also suggest that Pcdh-γ isoforms may associate with Pcdh-α and Pcdh-β isoforms. Should these Pcdhs share similar biochemical properties, the recognition specificities would be further diversified.

Thus, while the number of Pcdh isoforms pales in comparison to the number of Dscam1 isoforms, hetero-oligomerization of Pcdhs markedly increases the number of discrete binding specificities encoded by the locus. With the shared properties of clustered Pcdhs and Dscam1—isoform diversity, homophilic binding specificity, and stochastic combinatorial expression—Pcdhs have emerged as attractive candidates for regulating self-avoidance in vertebrates.

**Pcdh-γs Regulate Self-Avoidance and Self/Nonself-Discrimination in the Mouse Brain**

Lefebvre et al. (2012) set out to assess whether Pcdh-γ genes regulate self-avoidance in the mouse retina. They focused on the stereotyped relationship between dendrites of the same SACs and between dendrites of different SACs. SACs arborize extensively within specific laminae (designated S2 and S4) in the IPL of the mouse retina. SAC dendrites make contacts with dendrites of other SACs and form transient contacts with sister dendrites at early postnatal stages. These self-contacts are promptly eliminated to generate a radial arbor with minimal overlap among self-dendrites and no autapses. Thus, by these criteria, SACs distinguish between self and nonself.

By using the Cre-Lox system, Lefebvre and colleagues selectively deleted all 22 variable domains of the Pcdh-γ locus in the developing retina and demonstrated that SAC processes from the same cell crossed each other extensively, effects that looked remarkably similar to the removal of Dscam1 from da neurons...
They also demonstrated that single arbitrarily chosen isoforms rescued the mutant phenotype and that expression of the same isoform in neighboring SACs reduces the overlap between them (Figure 3g, h). Furthermore, although single isolated wild-type SACs exhibited self-avoidance in vitro, dendrites from single mutant SACs formed clumps. These data indicate that Pcdh-γs act in a cell-autonomous fashion and that they mediate interactions between Pcdh-γ isoforms on dendrites of the same cell. These data further argue that, as with Dscam1, self-avoidance in SACs does not rely on a specific isoform, but rather requires that isoform use differs among neighboring cells.

Genetic analyses have also revealed that subsets of the Pcdh-γs, the A/B Pcdh-γs, and C Pcdh-γs have diverse functions. Deletion of all 22 Pcdh-γs led to massive cell death. Self-avoidance is not an indirect effect of this cell death because cell death, but not the self-avoidance phenotypes, was suppressed in Bax−/− mice (Lefebvre et al. 2012). Furthermore, this cell death was seen in animals lacking C3–C5 but not in animals with deletions of A1–A3 (Chen et al. 2012). Thus, these data argue that variable Pcdh-γs are essential for self-avoidance in SACs, whereas the constant domains are required for cell survival. Finally, Lefebvre et al. (2012) also demonstrated that Pcdh-γs promote self-avoidance between dendrites of cerebellar Purkinje cells. Future experiments will be important to determine whether the Pcdhα and Pcdhβ proteins also contribute to self-avoidance in these or other neurons. In summary, clustered Pcdh-γs allow neurites of different cells to discriminate between self and nonself, thereby promoting self-avoidance.

CONCLUDING REMARKS

As proposed approximately 30 years ago, studies over the past 5–10 years have demonstrated that self-avoidance plays a widespread role in patterning axons and dendrites in both vertebrates and invertebrates. A common molecular strategy underlies this process, albeit through the utilization of different large families of cell recognition molecules. In each system, neurons express multiple isoforms of isoform-specific homophilic cell recognition molecules. Although the mechanisms by which specific isoforms are selected for expression within each cell remain unclear, each cell appears to express a unique population of isoforms endowing it with a cell surface identity significantly different from its neighboring neurons. This difference and the isoform-specific binding properties of different isoforms endow each neurite with the ability to discriminate between a sister neurite and the neurite of other cells, even cells of the same type. Through activation of a common signaling domain, via homophilic binding, sister neurites are repelled from one another. Given the complexity of both the insect and mammalian nervous systems, the diverse and highly specific binding properties of different isoforms may also contribute to other aspects of neuronal patterning in different developmental contexts.

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