A Novel Mouse Dscam Mutation Inhibits Localization and Shedding of DSCAM

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Abstract

The differential adhesion hypothesis of development states that patterning of organisms, organs and tissues is mediated in large part by expression of cell adhesion molecules. The cues provided by cell adhesion molecules are also hypothesized to facilitate specific connectivity within the nervous system. In this study we characterize a novel mouse mutation in the gene Dscam (Down Syndrome Cell Adhesion Molecule). Vertebrate DSCAM is required for normal development of the central nervous system and has been best characterized in the visual system. In the visual system DSCAM is required for regulation of cell number, mosaic formation, laminar specificity, and refinement of retinal-tectal projections. We have identified a novel mutation in Dscam that results in a single amino acid substitution, R1018P, in the extracellular domain of the DSCAM protein. Mice homozygous for the R1018P mutation develop a subset of defects observed in Dscam null mice. In vitro analysis identified defects in DSCAM R1018P localization to filopodia. We also find that wild type DSCAM protein is constitutively cleaved and shed from transfected cells. This secretion is inhibited by the R1018P mutation. We also characterized a novel splice isoform of Dscam and identified defects in lamination of type 2 and type 6 cone bipolar cells in Dscam mutant mice. The identification and characterization of partial loss of function mutations in genes such as Dscam will be helpful in predicting signs and symptoms that may be observed in human patients with partial loss of DSCAM function.

Introduction

Identifying the mechanisms by which cells differentiate into complex tissues is a central goal of developmental biology. The nervous system is a particularly difficult and exciting system in which to study development because of its complexity. The nervous system is composed of a very large number of cell types that make specific connections to a limited number of other cell types. Strong evidence suggests that the generation of distinct neural cell types is mediated through differential expression of transcription factors. These transcription factors execute expression of genetic programs that specify cell type. The specificity of connections that neurons make is then mediated by the production and recognition of extracellular cues. For example, netrins guide axons to their targets through interactions with various receptors [1].

Much initial work directed at understanding how connectivity within the nervous system develops focused on a simple system, the neuromuscular junction, and proteins such as Agrin and Musk that are essential for innervation of skeletal muscle [2,3,4]. The retina is another popular model system for understanding neural connectivity. The retina offers a more complicated system in which to study connectivity, containing a variety of neuron-neuron synapses, electrical synapses and organized circuits. The limited number of cell types and availability of transgenic models and antibody reagents, has also made the retina a focus of developmental neurobiologists. The retina is organized in both a top to bottom vertical field and a horizontal dorsal-ventral-lateral-medial field. Vertically the retina is organized into circuits that collect, process and transmit visual information to the rest of the brain. These circuits are specialized to detect different aspects of vision, such as color, movement and edges. Many types of retinal neurons are spaced in a non-random fashion across the horizontal plane of the retina. This spacing, referred to as mosaic spacing, is thought to ensure that a given portion of the visual world is sampled by most or all of the aforementioned specialized circuits [5].

Identification of genes that specify spacing and connectivity of retinal neurons has begun to produce a more complete picture of how the retina is organized. A combination of molecules acts to guide neurons towards making appropriate contact through differential adhesion. Differential adhesion involves the production of both adhesive cues and repulsive or indifference cues. Neuroligins and neurexins are among the best-characterized adhesive cues and their differential expression at the proto-
synapses of pre and postsynaptic cells help to facilitate the specificity of neural connectivity [6]. Differential adhesion also involves cues that specify avoidance, indifference or repulsion. The semaphorins and plexins, for example, guide the targeting of neurites within the retinal innerplexiform layer to specific depths. Semaphorins and plexins also prevent fasciculation of the neurites of some cell types in which they are expressed [7,8,9]. Proteins such as MEGF10 and MEGF11 also mediate avoidance and are required in order to facilitate horizontal spacing of cholinergic and horizontal cells within the retina, while the gamma-protocadherin complex mediates isoneuronal avoidance between the processes of a single cell [10,11].

The immunoglobulin superfamily adhesion molecules Dscam and Dscam11 also function in preventing adhesion [12,13]. Dscam and Dscam11 are required for multiple aspects of retinal development, including mosaic patterning of cell types, prevention of fasciculation, regulation of cell number and laminar organization. DSCAM is produced in a large number of spatially overlapping cell types that all independently require DSCAM to prevent adhesion, but maintain their homotypic identity in its absence, in that they adhere to like cells. This suggests that the simple and elegant model proposed for MEGF protein function, in which a homophilic adhesion molecule mediates avoidance of cells within a cell type from like cells, is insufficient to explain DSCAM function. Current models therefore invoke DSCAMs acting as part of a larger adhesion code. In this model some as yet unidentified molecules act as cell type identifiers, while DSCAMs are required to prevent these cell types from adhering homotypically [14].

In this study we characterize function of the DSCAM protein in retinal development using a novel Dscam point mutation. We find that the mutation, which results in a single amino acid substitution, causes a subset of the Dscam null phenotypes. Through in vitro characterization we find that DSCAM is targeted to growth cone filopodia and post-translational cleaved. We also find that the ectodomain of the protein is shed. The point mutation allele blocks targeting to filopodia and secretion. In vivo, the mutant protein accumulates in the cell body or retinal neurons suggesting that the mutation prevents proper targeting of the protein. Our findings suggest a model in which wild type DSCAM can act as a diffusible ligand.

Results

A Dscam Point Mutation

A spontaneous recessive neurological mutation, nm2122, arose at The Jackson Laboratory on an inbred stock of C3H/Smm.C-Pkd.<scid> mice. Nm2122 presented an array of mutant phenotypes, including kyphosis, hydrocephalus and apparent vestibular defects (Figure 1 A–C). Based on the presentation of these phenotypes, and their initial mapping to the distal end of Chromosome 16, a complementation test was established between nm2122 and Dscam2J (nm992), a mutant mouse line that carries a loss of function allele of Dscam [15,16]. Out of 26 pups, five had phenotypes similar to that of nm2122 and Dscam2J, indicating that Dscam2J failed to complement nm2122 and that the mutations are allelic.

The nm2122 Dscam open reading frame was sequenced from whole brain cDNA and a single nucleotide substitution, guanine to cytosine at nucleotide 1018 in place of the wild type arginine in the second fibronectin domain of DSCAM (Figure 1E). The nm2122 mutation will be henceforth referred to as Dscam2J, because it is the third spontaneous Dscam mutation identified at The Jackson Laboratory, while the mutant protein will be referred to as DSCAMR1018P. A PCR RFLP genotyping technique was developed to genotype Dscam2J mice. Primers amplify the region containing the Dscam2J mutation from genomic DNA, which is then digested with the enzyme BsUI. The Dscam2J mutation destroys a BsUI site, and DNA amplified from mutant alleles retains a large undigested band after digestion, whereas DNA products amplified from wild type alleles result in a digested product (Figure 1E). Western blot analysis (WBA) was performed to determine if DSCAM protein was made in Dscam2J mutant mice. WBA of protein extract from wild type postnatal day 15 (P15) cerebellum resulted in a bright band of approximately 220 kD in size and a faint band of slightly smaller size. A single band of approximately 220 kD in size was detected in Dscam2J extracts, while no band was observed in protein extracts from Dscam3J mice (Figure 1G). Similar WBA results were obtained from these genotypes using protein extracts generated from retina, cortex and olfactory bulb (data not shown).

Characterization of Mutant Phenotypes in Dscam3J Mice

The inbred line that the Dscam3J allele arose on carries the recessive retinal degeneration allele of Pde6b, rd1. Pde6brd1 results in rapid degeneration of photoreceptors around the time of eye opening. In order to examine retinal structure of Dscam3J mice, the line was crossed to an inbred C3H line that carries a wild type allele of Pde6b [17]. Other lines of Dscam mutant mice have disrupted laminar specificity, arborization and spacing of retinal neurons. We assayed these phenotypes in Dscam3J mice. Dscam3J mice were used as negative controls because they are on a similar genetic background and because Dscam4017 mice, the first published Dscam mouse mutant, make a small amount of residual protein as a result of an alternative splice form (Figure S1) [12]. We first assayed gross retinal lamination by staining sections of wild type, Dscam3J and Dscam3J retina with hematoxylin and eosin (H&E). The Dscam3J allele resembled the protein null Dscam3J allele in that the size of the inner plexiform, inner nuclear and retinal ganglion cell layers were expanded. Unlike the Dscam3J inner nuclear layer, which contains fasicles of processes, the Dscam3J inner nuclear layer is evenly laminated (Figure 2 A–C).

This suggested that the amacrine cells of the Dscam3J inner nuclear layer may be less disrupted than the amacrine cells of the Dscam3J retina. We therefore stained amacrine and ganglion cell populations in whole retinas to examine their neurite arborization and soma spacing. Arborization and soma spacing defects were observed in intrinsically photosensitive retinal ganglion cells (ipRGCs), a population that is highly sensitive to Dscam dosage [18,19] (Figure 2 D–F). Less extensive defects were observed in the arborization and spacing of dopaminergic amacrine (DA) cells in Dscam3J retinas compared to Dscam3J retinas (Figure 2 G–I). An increase in the incidence of juxtaposed DA cells was observed in the Dscam3J retina compared to wild type or random simulations matched for cell number (Average 5.15 pairs per retina in simulations versus 19 in Dscam3J retinas T-Test = 0.01). Loose fasciculation of DA cell neurites was occasionally observed in the Dscam3J retina (Figure 2J; arrows). Similar defects in cell number and spacing were observed in bNOS amacrine cells (Figure 2K and L). bNOS positive amacrine cells also appeared hypertrophied in the Dscam3J mutant retina, in a similar fashion to the hypertrophy observed in some Dscam1L-deficient cell types [13]. Therefore, defects in cell number are conserved between Dscam3J and other Dscam mutant alleles, but a lesser disruption in arborization and spacing of amacrine cells was observed.

Neurite lamination defects are observed in Dscam3J mutant mice. Antibodies recognizing amacrine, ganglion and bipolar cell
A spontaneous mutation, nm2122, occurred at The Jackson Laboratory and exhibited overt phenotypes, including a large dome shaped head and muscle stiffness, similar to previously characterized Dscam mutants. B and C, nm2122 mutant mice develop enlarged central and lateral ventricles compared to controls (arrows). D, After complementation tests with another Dscam mutant, NM992 (Dscam<sup>2J</sup>), failed, the Dscam open reading frame was sequenced and a single nucleotide substitution was found resulting in substitution of proline at 1018 in place of the wild type arginine. The nm2122 mutation will henceforth be referred to as Dscam<sup>2J</sup>. E, The Dscam<sup>2J</sup> mutation is located in the second fibronectin domain (arrow). F, The mutation destroys a recognition site for the enzyme BstUI, allowing wild type, heterozygous and homozygous mutants to be identified based on PCR of the mutation-containing region followed by subsequent restriction enzyme digest. The wild type allele is digested by BstUI resulting in two bands of close to equivalent size, while the mutant allele remains intact. G, Western blot analysis of wild type, Dscam<sup>2J</sup> and Dscam<sup>2J</sup> mutants. A polyclonal antibody to the N-terminus of DSCAM recognizes a single band of approximately 220 kD and a slightly smaller band in retinal extracts prepared from wild type mice while a single band of 220 kD was detected in Dscam<sup>2J</sup> extracts. No protein product is observed from retina (not shown) or brain extract prepared from Dscam<sup>2J</sup> mice. The scale bar in (C) is equivalent to 1 cm.

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Figure 1. Amino Acid substitution R1018P is genetic basis of nm2122. A, A spontaneous mutation, nm2122, occurred at The Jackson Laboratory and exhibited overt phenotypes, including a large dome shaped head and muscle stiffness, similar to previously characterized Dscam mutants. B and C, nm2122 mutant mice develop enlarged central and lateral ventricles compared to controls (arrows). D, After complementation tests with another Dscam mutant, NM992 (Dscam<sup>2J</sup>), failed, the Dscam open reading frame was sequenced and a single nucleotide substitution was found resulting in substitution of proline at 1018 in place of the wild type arginine. The nm2122 mutation will henceforth be referred to as Dscam<sup>2J</sup>.
Figure 2. *Dscam*" mutation reproduces some aspect of *Dscam* null retina. A–C, Retinal sections from wild type, *Dscam*" and *Dscam*" mice were stained with hematoxylin and eosin. In the wild type retina (A) the three cellular layers are neatly stacked and the synapse containing plexiform

ONL, OPL, INL, IPL, RGL

Melanopsin

Tyrosine Hydroxylase

TH, bNOS

Figure 2. *Dscam*" mutation reproduces some aspect of *Dscam* null retina. A–C, Retinal sections from wild type, *Dscam*" and *Dscam*" mice were stained with hematoxylin and eosin. In the wild type retina (A) the three cellular layers are neatly stacked and the synapse containing plexiform
layers do not intrude within the cellular layers. B. Cell number is increased in the Dscam<sup>−/−</sup> mutant retina, with ectopic cells located in the inner plexiform layer, which projects into the inner nuclear layer. C. The Dscam<sup>−/−</sup> retina is hypercellular; however, cellular lamination is more neatly organized compared to the Dscam<sup>−/−</sup> retina. D-F. Retinal ganglion cell spacing and arborization is disrupted in both the Dscam<sup>−/−</sup> and Dscam<sup>−/−</sup> retina compared to wild type. G-I. Amacrine cell spacing and arborization is disrupted in the Dscam<sup>−/−</sup> mutant retina but this degree of disruption is not observed in the Dscam<sup>−/−</sup> mutant retina (I). J. Occasional loose fasciculation of Dscam<sup>−/−</sup> dopaminergic cell neurites was observed (arrows). K and L. Wild type and Dscam<sup>−/−</sup> retinas were stained with antibodies to bNOS, to detect bNOS-positive amacrine cells. The number of bNOS positive amacrine cells is increased in the Dscam<sup>−/−</sup> retina. The scale bar in (A-C) is equivalent to 132 μm. The scale bar in (I) is equivalent to 320 μm in D-I and K and L, and 100 μm in J.

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of bands at and below 45 kD (the expected size of the DSCAM C-terminus) in lysates from cells that had been transfected with the C-terminus of Dscam. No band was detected in lysates from untransfected cells. Two bands from lysates transfected with Dscam or myc/his tagged Dscam were detected. The larger of these bands matched the size of full length DSCAM. The second matched the size of the C-terminus of DSCAM. The smaller band from myc/his tagged Dscam transfected lysates was shifted up in size slightly, confirming that this band is a product of the Dscam transcript (Figure 5B, arrows). The presence of multiple bands produced from a single cDNA construct, and their respective sizes, suggested that DSCAM was being cleaved post-translationally into N-terminal and C-terminal fragments. Many cell adhesion molecules are processed by cleavage of their ectodomain and cytoplasmic domains after they reach the plasma membrane. Further studies were therefore conducted to investigate the mechanism by which DSCAM is processed. We first determined whether DSCAM is shed into cell culture media. WBA was performed on conditioned media (CM) from transfected and untransfected cells to determine if DSCAM was shed. N-terminal DSCAM antibodies labeled a band in conditioned media from transfected cells corresponding to the size of the N-terminus of DSCAM, while C-terminal antibodies did not recognize a specific band, indicating that the N-terminal portion of DSCAM can be shed. We also found that the amount of shed DSCAM protein increased over time (Figure 5C). To confirm that secretion was the result of an active process, occurring after DSCAM localization to the plasma membrane, and not the result of cell rupture, cells were treated with brefeldin-A (BFA), to block trafficking to the plasma membrane. Treatment with BFA effectively eliminated production of the shed isoform of DSCAM and production of the smaller C-terminal fragment of DSCAM, indicating that the protein must reach the plasma membrane before cleavage can occur (Figure 5D).

We next introduced the R1018P mutation into the Dscam expression construct and assayed expression by WBA. Expression of Dscam<sup>R1018P</sup> resulted in production of full length DSCAM and the small C-terminal band (Figure 5E). WBA of DSCAM<sup>R1018P</sup> cell lysates indicated that both bands detected by the N-terminal antibody were present; however, very little DSCAM<sup>R1018P</sup> was detected in conditioned media produced by the same cells (Figure 5E). The extent of DSCAM secretion was quantified by comparing the ratio of full length DSCAM detected in cell lysates using the N-terminal DSCAM antibody to the amount of DSCAM in conditioned media produced by the same cells. A significant decrease was found in the amount of DSCAM shed into conditioned media when comparing R1018P DSCAM to wild type DSCAM (Figure 5G).

Cells expressing Dscam or Dscam<sup>R1018P</sup> were stained with N-terminal antibodies to assay if a difference in localization could account for the Dscam<sup>−/−</sup> phenotype and lack of DSCAM<sup>R1018P</sup> secretion. DSCAM was localized to the filapodia of N2A growth cones, while DSCAM<sup>R1018P</sup> was localized to vesicles within the growth cone, with only small amounts of the protein visible in the filopodia (Figure 6 A and B). DSCAM<sup>R1018P</sup> is located in the second fibronectin repeat of DSCAM. We generated a Dscam expression construct that lacks the second and third fibronectin repeats to assay if this domain is required for localization of DSCAM to filopodia. DSCAM lacking the middle two fibronectin repeats localized to filopodia, suggesting that the R1018P mutation does not prevent localization to the filopodia by disrupting the normal function of these repeats (Figure 6 C-E).

The R1018P mutation occurs immediately before a predicted beta-strand, based on the structure of a type III fibronectin repeat from DSCAML1, which shows a high degree of homology [20]. Based on the abnormal localization in vitro we speculated the mutation might get trapped in the cell body in vivo. We therefore compared the localization of DSCAM and DSCAM<sup>R1018P</sup> in sections of retina (Figure 6 F and G). While the DSCAM antibody used shows some nonspecific background, a clear increase in DSCAM<sup>R1018P</sup> immunoreactivity is observed in the cell body of neurons in the retinal ganglion cell layer in Dscam<sup>−/−</sup> sections compared to wild type DSCAM protein in wild type sections. Therefore the DSCAM<sup>R1018P</sup> allele is not localized normally, but wild type localization in vitro is independent of the fibronectin repeat where the mutation occurs. This likely reflects larger scale changes to protein structure, which may be unsurprising given the unique conformation of proline and its effect on protein structure. To test this speculation we used molecular dynamics simulation to model the effect of the R1018P mutation on the structure and dynamics of the fibronectin domain. Figure 7A shows the separation between the alpha carbon at the mutation site and two alpha carbons on neighboring loops as a function of simulation time. Results show that the mutation increases the distance between these carbons, consistent with the idea that the mutation is destabilizing the protein domain. Figure 7 B and C shows the predicted structure of the wild type and R1019P DSCAM fibronectin domain based on homology modeling using a Dscam-like structure as a template. The alpha carbon of amino acid 1018 is shown as a green sphere and the other colors represent the amount of fluctuation in the structure (blue = smallest fluctuations, red = largest fluctuations). Results show that the mutation increases the dynamic fluctuations around the mutation site and is consistent with the idea that the mutation is destabilizing the protein domain.

Discussion

In this study we describe a novel mutant allele of the mouse Dscam gene, Dscam<sup>−/−</sup>. Dscam is one of many cell adhesion molecules that contributes to retinal development. Dscam stands out with respect to the larger number of phenotypes observed in Dscam mutant mice, including arborization, cell number and neurite laminar specificity defects. Understanding the relationship of defects observed in Dscam mutant mice, together with normal function of the DSCAM protein, remains a challenge. The results of this study will facilitate a better understanding of these processes.

Dscams have been most thoroughly studied in Drosophila, and mediate a similar range of processes as their vertebrate homo-
logues, which lack the extensive alternative splicing observed in Drosophila Dscam1 [21,22,23,24,25,26,27]. Studies of vertebrate Dscams have identified at least three potential functions for these genes that overlap with functions demonstrated for their homologues in Drosophila. The first of these phenotypes, described in zebrafish, is a role in axon outgrowth [28]. Mouse DSCAM was subsequently found to bind the ligands netrin and draxin, although identifying guidance phenotypes has been uncertain [29,30,31,32]. Next, gain of function and loss of function in chick suggest that Dscams are required for neurite laminar specificity [33]. Studies using mouse mutants have identified function in arborization, regulation of cell number and facilitating mosaic distribution of cell soma [12,13].

Initial studies of mouse Dscam mutants found that wild type Dscam is required for normal spacing and arborization of amacrine cells. The different laminar targeting of these cell types suggested that DSCAM might act directly like MEGF proteins to facilitate avoidance. The finding that most retinal ganglion cells also require DSCAM to avoid getting entangled in like dendrites indicated that this model was too simple, because of the significant overlap of retinal ganglion cell dendrites. This suggested that mouse Dscams are acting as part of a larger identity code, and do not specify cell type [13]. Conditional deletion of Dscam confirmed that the protein acts to prevent fasciculation by acting within and not between retinal cell types and through homotypic binding, in that the null phenotype is dominant within cell types, that is mutant...
cells will entangle homotypic wild type cells within the same retina [34]. All of these mechanisms in mouse retina are consistent with DSCAM acting as a homophilic transmembrane cell adhesion molecule.

In this study we describe a new mutant allele of Dscam, further characterize the protein and show that DSCAM can be either shed or secreted. The Dscam3J allele shows a mixture of wild type and mutant phenotypes (Table 1). Unlike Dscam2J, in which no protein product was observed in retina or brain, Dscam3J makes a full-length protein. A simple explanation for the intermediate phenotypes observed in the Dscam3J allele is that the Dscam3J mutation is functionally a hypomorph, as a result of defective protein trafficking or reduced protein activity. Limited dopaminergic amacrine cell and ipRGC spacing disruption and cell number increases are previously described Dscam dosage phenotypes and the presence of these phenotypes in the Dscam3J retina is consistent with the allele acting as a hypomorph.

Both wild type and DSCAMR1018P get cleaved into two products in vitro; however, DSCAMR1018P is shed in conditioned media at very low levels compared to wild type DSCAM. The lack of DSCAMR1018P secretion seems best explained as the result of localization defects. Evidence for secretion of wild type DSCAM was found in vitro, where it is clearly secreted, and also in vivo, where the detection of a smaller isoform of DSCAM would be consistent with shedding. Secretion of fly DSCAM has also been reported but potential functions in neural development have not been identified [35]. In the context of DSCAM's role in preventing adhesion, cleavage and secretion may be unsurprising as the tight binding of DSCAM isoforms must be overcome in order to facilitate repulsion, in flies, or prevent adhesion, in mouse, yet it has not been well documented [36]. Secretion and anchoring of DSCAM to cells in a similar fashion to the manner in which the axon guidance molecule Slit has recently been reported to act could explain why some cell types that do not express Dscam have a
Figure 6. Dscam<sup>−/−</sup> mutation inhibits filopodial localization of DSCAM. A–C, Dscam expression constructs were transfected into N2A Cells. A, Full length canonical DSCAM is abundant in the golgi-ER, where it is translated and trafficked, along the membrane and at the tips of filopodia. B, DSCAM<sup>R1018P</sup> is translated and trafficked in and from the golgi-ER and appears in trafficking vesicles within growth cones but does not appear to be localized to the filopodia. C, The localization defect in DSCAM<sup>R1018P</sup> is not a result of disrupted fibronectin domain function, as deletion of the second and third fibronectin domain of DSCAM does not interfere with filopodial localization. D and E, DSCAM or DSCAM<sup>R1018P</sup> were transfected into N2A...
strong laminar specificity phenotype in Dscam mutant mice [37]. New studies taking advantage of newly developed conditional gain of function Dscam mouse alleles to complement existing mutant alleles will permit identification of potential roles of shed DSCAM in vivo by combining the ability to assay loss of function with conditional over or ectopic expression.

The contribution of cell adhesion molecules and the recognition they encode remains an emerging story in developmental neurobiology. In this study we identify a localization and secretion-deficient point mutant allele of mouse Dscam and use this model to better characterize the role of DSCAM in neural development. This resource will aid in efforts to understand how neural patterning is facilitated by the balance of adhesion and avoidance.

Materials and Methods

Animal Care and Ethics

All protocols were performed in accordance with the University of Idaho Institutional Animal Care and Use Committee which draws from NIH guidelines to ensure that animal suffering is minimized. Mice were fed ad libum under a 12-hour light/dark cycle. Mice taken for study were first deeply anesthetized with tribromoethanol (500 mg/kg). Blood was flushed out of vessels by cardiac perfusion. Following cardiac perfusion mice were decapitated and tissue was collected.

Figure 7. Molecular dynamics simulations suggest that the R1018P mutation destabilizes the DSCAM fibronectin domain. (A) Separation between the alpha carbon at the mutation site residue and two alpha carbons on neighboring loops as a function of simulation time. (B) Predicted structure of the wild type domain. The alpha carbon of amino acid 1018 is shown as a green sphere and the other colors represent the amount of fluctuation in the structure (blue = smallest fluctuations, red = largest fluctuations). (C) Same as panel B, but for the R1018P mutant.

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Table 1. Phenotypes in Dscam<sup>3</sup> mice.

| Phenotype          | Wild type | Dscam<sup>3</sup> | Dscam<sup>3</sup> |
|--------------------|-----------|-------------------|-------------------|
| Dendrites          | Arborized | Fasciculated      | Mixed             |
| Soma Spacing       | Spaced    | Clumped           | Weak Clumping     |
| Cell number        | Wild type | Overabundant      | Overabundant      |
| Neurite lamination | Organized | Disorganized      | Organized         |
| LGN refinement     | Wild type | Abnormal          | Like Wild type    |
| In vitro secretion | Yes       | N.A.              | No                |
| In vitro targeting | Filopodia | N.A.              | No                |

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Protein Extraction and Western Blot

Protein was extracted from tissue and separated into hydrophilic and hydrophobic fractions using mem-PER Eukaryotic Membrane Protein Extraction Kit according to instructions (ThermoScientific, Rockford, IL). Both fractions were found to contain DSCAM or DSCAM<sup>R</sup> and the cytoplasmic fraction was used because it contained more protein. All reagents included the addition of 0.005M EDTA and Halt protease and phosphatase inhibitor cocktail (ThermoScientific, Rockford, IL). Western blotting was performed as described previously [38] with the following modifications. Tissue protein samples were diluted two-fold to prevent band distortion. Samples were incubated in 25% loading buffer for 1 hour at room temperature. Samples were not boiled in order to avoid agglutination of larger membrane proteins. Electrophoresis was performed on hydrophilic and hydrophobic fractions using 7% polyacrylamide gels. Transfer of proteins to immobilon-P membranes (0.45 um; Millipore, Billerica, MA) was carried out for 19–22 hours using 30 volts at 4°C. Methanol-free transfer buffer containing 0.037% SDS was used for the wet transfer to prevent precipitation of larger membrane proteins. All reagents included the addition of 0.005M EDTA for 15 minutes. Samples were neutralized with an equal volume of Tris Cl, pH 5.0. DNA was added to OneTaq Hot Start 2X Master Mix with standard buffer (New England Biolabs, Ipswich, MA) and run with the following PCR cycle: 94°C 2 minutes, 38 cycles of 94°C 30 seconds, 53.5°C 30 seconds, 72°C 25 seconds, followed by a final 72°C incubation for two minutes. Primers used were 5’TGG GCC GCG TTA TGA TC T 3 and 5’ GTG GTG TGG TCG ATA CTG ATG 3’. PCR products were incubated at 60°C for 2 hours in an equal volume of digestion mix containing 1 μl BstUI restriction enzyme, 10% Buffer 4 and then run on a 2% agarose gel for approximately 1.5 hours.

cDNA Synthesis and RT-PCR

Total brain was homogenized in trizol reagent and RNA was isolated according to manufacturer’s instructions. A total of 1 μg total RNA was used to synthesize cDNA using a superscript III kit (Invitrogen) according to manufacturer’s instructions.

Brain Section Staining

Brains were fixed in 4% paraformaldehyde overnight and sectioned at 150 μm with a vibratome. Brain sections were stained with 0.1% cresyl violet and 0.3% acetic acid for five minutes and then rinsed in PBS.

Retina Staining

Eyes were enucleated following cardiac perfusion and hemisection. The posterior half of the eye was incubated in 4% paraformaldehyde for 50 minutes (DSCAM staining), overnight (for melanopsin staining) or 4–6 hours (all other staining) followed by three washes in PBS. For sectioning, retinas were isolated from the posterior half of the eye and either dehydrated and embedded in paraffin or equilibrated in 30% sucrose for one hour and then frozen in OCT. Sections were cut with a cryostat or microtome at 10 μm onto superfrost plus slides. Paraaffin sections were rehydrated and stained with hematoxylin and cosin. Frozen sections were blocked in 7.5% normal donkey serum and 0.1% Triton X-100 in PBS (block). Primary antibodies were diluted in block and incubated overnight at 4°C. Primary antibodies were washed three times in PBS for ten minutes. Secondary antibodies were diluted in block and 500 μl was applied over a given slide and incubated at room temperature for two hours. Slides were then washed three times for fifteen minutes in PBS. The second wash contained 1 μl DAPI solution. Whole retinas were stained in a similar fashion except that the block contained 0.4% Triton and incubation of primary antibodies was carried out over four days, while secondary antibodies were incubated at 4°C for four days.

Antibodies

Goat-anti-DSCAM (R&D Technologies 1:500 for WBA and 1:100 for IHC), rabbit-anti-melanopsin (generously gift of Ignacio Provencio, Uniformed Services University of the Health Sciences, at 1:10,000), mouse-anti-tyrosine hydroxylase (Leica 1:50), rabbit-anti-bNOS (Sigma Aldrich 1:5,000), goat-anti-ChATp (Chemicon; 1:500), mouse-anti-PKCa (Santa Cruz Biotechnology 1:500), rabbit-anti-DSCAM (generous gift of Robert Burgess, The Jackson Laboratory) and mouse-anti-synaptotagmin2 (ZIRC; 1:500). Fluorescent secondary antibodies were obtained from Jackson Immuno Research and were used at 1:500.

LGN Labeling

Mice were anesthetized with isofluorane gas. Following anesthesia, pupils were dilated by instillation of 1 drop of tropicamide 5%. One drop of tetracaine 1% was then administered for local anesthesia. Levels of anesthesia were monitored by brushing the cornea of the mouse with a fine paintbrush. Paralube was applied to eyes, removed during injection and reapplied following completion of injection in order to prevent eyes from drying out. Intravitreal injections were made using a 30 gauge Hamilton syringe. The eyes were visualized using a stereo dissecting microscope. A puncture was made in the eye posterior to the anterior/posterior boundary using the 30 gauge needle, which was inserted into the posterior chamber of the eye at a 45 degree angle in order to avoid the lens. Intravitreal injections of cholera toxin-β subunit (CTβ), conjugated to Alexa Fluor 488 (green) or Alexa Fluor 594 (red) were performed. CTβ (Invitrogen) conjugated to Alexa Fluor 488 (green label) was injected into one eye, and CTβ conjugated to Alexa Fluor 594 (red label) was injected into the other eye (2–3 μl; 0.5% in sterile saline). Placement of the needle within the eye and release of dye was monitored by directly viewing the posterior chamber of the eye through the stereomicroscope. Post-injection mice were placed in
an incubator set to 30°C, until recovery and housed for 24 hours. 24 hours after injection, mice were euthanized and brain tissue was harvested and fixed overnight in 4% paraformaldehyde (PFA), cryoprotected in 30% sucrose, then sectioned coronally at 150 μm on a vibratome, mounted onto slides, and coverslipped with Vectashield (Vector Laboratories). Slides were imaged with an Olympus spinning disk microscope.

Expression Constructs
The open reading frame of Dscam was PCR amplified out of total mouse brain cDNA and cloned into the EcoRI site of pCAG to generate pCAG-Dscam. A Myc/His tag was added to the C-terminus to generate pCAG-DscamMyc/His. Site directed mutagenesis was used to introduce the R1018P mutation into pCAG-Dscam to generate pCAG-DscamR1018P. Site directed mutagenesis was used to delete the second and third fibronectin domains of Dscam from pCAG-Dscam to generate pCAG-Dscam del17-3. All constructs were sequenced to ensure the accuracy of the sequence and any subsequent changes.

Cell Culture
N2A and HeLa cells were acquired from American Type Culture Collection and maintained in DMEM with glutamax (Invitrogen) supplemented with 10% fetal bovine serum. Cells were split by releasing cells using TrypLE (Invitrogen). Cells were transfected with Fugene6 reagent at a ratio of 3 μg Fugene to 1 μg DNA (Roche). Brefeldin A (Sigma Aldrich) was administered at a dose of 2 μg/ml one hour after transfection. Cells were maintained for twenty four or forty eight hours after transfection and harvested for imaging or western blot analysis. For imaging, cells were seeded onto poly-D-lysine treated coverslips and stained as retinal sections were, except that primary antibodies were applied for two hours at room temperature. Mem-per reagent (Thermo Scientific) was used to extract protein from cells.

Molecular Dynamics Simulations
Initial coordinates for the wild type and mutant protein domains were obtained using SWISS-MODEL with Protein Data Bank structure 1VA9 as a template [39]. Simulations were performed using the GROMACS 4.5.5 and the GROMOS 53A6 force field [40]. The charge was neutralized using sodium and chloride ions with an ion concentration of 0.15 mol/L. All water molecules were obtained using SWISS-MODEL with Protein Data Bank structure 1VA9 as a template [39]. Simulations were performed using the LINCS algorithm. The temperature was maintained at 300 K using Langevin dynamics, the pressure was maintained at 1.0 atm using the Parrinello–Rahman algorithm. Van der Waals interactions were truncated using a cutoff of 1.4 nm, and electrostatics were treated using reaction-field with a cutoff of 1.4 nm. Both protein systems were first minimized using steepest descent for 1000 steps, followed by 1.0 ns of simulation with heavy atom restrained, followed by 1.0 ns of unrestrained simulation. Production simulations were then performed for 100 ns for each protein, generating a trajectory of conformation snapshots for each protein system. Protein fluctuations were determined by calculating the root-mean square fluctuations of the alpha carbons. This calculation is performed by first performing a least squares superposition of the structures in each trajectory and then computing the root-mean square deviation of the alpha carbons from their average positions.

Supporting Information
Figure S1 Alternative splicing of mouse Dscam. A. Western blot analysis was performed on p0 wild type and DscamR1018P mutant cytoplasmic protein extracts. Two bands were observed in wild type extracts, corresponding to the size of full length DSCAM and a slightly smaller band. DscamR1018P protein extracts had two faint immunopositive bands, both slightly smaller than the corresponding wild type bands. Similar results were obtained for membrane extracts except that the DscamR1018P protein bands were barely visible (data not shown). B, An alternative splice site was identified within exon 15 of canonical Dscam. The transcript made by use of the alternate acceptor site is not in frame and no corresponding protein was detected. C, In the context of the DscamR1018P mutation the alternate acceptor site regains the Dscam open reading frame before hitting a stop codon, resulting in the absence of 94 wild type amino acids and the substitution of 54 alternative amino acids.

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Author Contributions
Conceived and designed the experiments: PGF. Performed the experiments: PGF RDS SL BSH RPR FMY. Analyzed the data: PGF. Performed the experiments: SL BSH RPR. Contributed reagents/materials/analysis tools: PGF RWB. Wrote the paper: PGF.

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