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Neuronal Clustering and Fasciculation Phenotype in *Dscam*- and *Bax*-Deficient Mouse Retinas

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Abstract

Individual types of retinal neurons are distributed to minimize proximity to neighboring cells. Many of these same cell types extend dendrites to provide coverage of the retinal surface. These two cardinal features of retinal mosaics are disrupted, for certain cell types, in mice deficient for the Down syndrome cell adhesion molecule, *Dscam*, exhibiting an aberrant clustering of somata and fasciculation of dendrites. The *Dscam*-mutant mouse retina also exhibits excess numbers of these same cell types. The present study compared these two features in *Dscam*-mutant retinas with the *Bax*-knockout retina, in which excess numbers of two of these cell types, the melanopsin-positive retinal ganglion cells (MRGCs) and the dopaminergic amacrine cells (DACs), are also present. Whole retinas were immunolabeled for both populations, and every labeled soma was plotted. For the MRGCs, we found a gene dosage effect for *Dscam*, with the *Dscam*^{+/-} retinas showing smaller increases in cell number, clustering and fasciculation. Curiously, *Bax*^{-/-} retinas, showing numbers of MRGCs intermediate to those found in the *Dscam*^{-/-} versus *Dscam*^{+/-} retinas, also had clustering and fasciculation phenotypes that were intermediate to retinas with those genotypes. DACs, by comparison, showed changes in both the *Dscam*^{-/-} and *Bax*^{-/-} retinas that did not correlate with their increases in DAC number. The fasciculation phenotype in the *Dscam*^{-/-} retina was particularly prominent despite only modest clustering. These results demonstrate that the somal clustering and fasciculation observed in the *Dscam*-mutant retina are not unique to *Dscam*-deficiency, and are manifested distinctively by different retinal cell-types.

Keywords

melanopsin retinal ganglion cell; dopaminergic amacrine cell; inner plexiform layer; Voronoi domain; L function; retinal mosaic

Introduction

Retinal neurons minimize proximity to homotypic neighbors, establishing a patterning in the distribution of their somata. Additionally, their dendrites are typically distributed to achieve a characteristic degree of overlap for a given class of cell, ensuring a uniform coverage of the retinal surface (see Reese, 2008, for review). These two cardinal features of “retinal mosaics” have recently been shown to be disrupted in *Dscam*-mutant mice: their retinas show a conspicuous clustering in the distribution of certain types of retinal nerve cells, and a fasciculation of their processes (Fuerst et al., 2008; 2009). Heterotypic cell-types normally expressing *Dscam* do not appear to cluster nor fasciculate with one another in the mutant, indicating that the normal function of mouse *Dscam*, being a member of the Ig superfamily of cell adhesion molecules, is to mask adhesive interactions between homotypic neighbors that are unique to each type of cell (Fuerst and Burgess, 2009). Functional *Dscam* would appear to prevent such homotypic interactions mediating adhesion, allowing dendritic arbors to extend freely in the presence of like-type dendrites.

Two retinal cell-types that exhibit these features in the *Dscam*-mutant mouse are the intrinsically-photosensitive melanopsin-positive retinal ganglion cells (MRGCs) and the dopaminergic amacrine cells (DACs). In addition to having this clustered and fasciculated phenotype, however, both populations are more numerous in the *Dscam*-mutant retina (Fuerst et al., 2008; 2009). Although the reasons are still currently unclear, a lack of normal developmental cell death appears to be contributing to their overabundance. Curiously, we have observed that *Bax*-knockout mice, containing elevated numbers of various cell types due to disrupted apoptosis during development, also exhibit a clustered and fasciculated phenotype amongst their MRGC population. In order to understand better the relationships between these three phenotypic features (increases in cell number, the presence of cellular clustering and the abnormal fasciculation of processes), the present study has examined the mosaics of both MRGCs and DACs in mice deficient for *Dscam* or *Bax*. Specifically, we have generated high-resolution confocal images of the entire retina to show the nature of these phenotypes and their variation across the retina. We then determined the size of each population by counting every cell across the entire retina. Using the X, Y coordinates to identify the position of each cell, we subsequently examined the variability in intercellular spacing within each population by determining the coefficient of variation derived from the Voronoi tessellation of large sampled fields from each mosaic. Finally, we assessed the presence of cellular clustering in these same fields using the L function¹.

Materials and Methods

Animals

Mice heterozygous for a mutant *Dscam* allele, in which a 38bp deletion approximately halfway through the coding sequence results in a frame shift mutation and no detectable protein by immunostaining (Fuerst et al., 2008), were obtained from The Jackson Laboratory and bred together to produce *Dscam*-heterozygous and *Dscam*-mutant mice (herein referred to as *Dscam*^{+/+} and *Dscam*^{-/-} mice, respectively). *Bax*-knockout mice (B6.129 x 1-*Bax*^{tm1Sjk/J}; herein referred to as *Bax*^{-/-}) had previously been obtained from The Jackson Laboratory and backcrossed for at least ten generations onto a C57BL/6J background. Littermate control mice were obtained from each litter. Because they did not differ significantly in their total number of DACs nor MRGCs, they have been combined as a

¹A preliminary report from another laboratory describing the clustering and fasciculation phenotypes amongst MRGCs in the *Bax*^{-/-} retina was presented at the 2009 Society for Neuroscience meeting (McNeill et al., 2009). A preliminary report of the work described in the present study was presented at the 13th Annual Vision Research Conference on Retinal Ganglion Cells: Development, Function, and Disease (Keeley et al., 2010).

single “wildtype” control group below. All breeding was performed in the Animal Resource Center at the University of California, Santa Barbara. To obtain retinas for immunofluorescence, mice were euthanized with sodium pentobarbital (120 mg/kg, i.p.), and perfused intracardially with 0.9% saline followed by 4% paraformaldehyde in 0.1M sodium phosphate buffer (pH 7.2 at 20°C) for 15 min. All three *Dscam*^{-/-} mice were 16 days of age, as were three of the *Dscam*^{+/-} mice. All other mice used were a minimum of 30 days of age, averaging 75 days. The number of mice analyzed in each group is indicated in figures 2a and 6a. (Because all of the quantifications described below are based on either total cell number or upon scale-independent spatial statistics, they are not impacted by variation in retinal size due to maturational differences). Eyes were dissected and then fixed by immersion for an additional 15 min in 4% paraformaldehyde. Each retina was dissected taking care to include its entire extent, with four radial relieving cuts extending roughly half-way to the optic nerve head being made to allow the retina to lie flat.

To obtain retinas for quantitative PCR, mice were given a lethal dose of sodium pentobarbital and eyes were removed and immediately transferred to cold phosphate-buffered saline (PBS). Retinas were then dissected and stored in RNAlater (Ambion, catalog #AM7020) until RNA could be extracted. Three mice were analyzed per genotype and all mice were taken at postnatal day 24. All experiments were conducted under authorization by the Institutional Animal Care and Use Committee at UCSB and in accord with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Immunofluorescence and Antibody Characterization

The populations of melanopsin retinal ganglion cells (MRGCs) and dopaminergic amacrine cells (DACs) were identified in each retina by immunofluorescence using rabbit polyclonal antibodies to melanopsin (1:10,000; courtesy of Ignacio Provencio, University of Virginia) and sheep polyclonal antibodies to tyrosine hydroxylase (1:10,000; Millipore, catalog #AB1542), respectively (Table 1). The immunogen that had been used to generate the affinity-purified polyclonal antibody to tyrosine hydroxylase (TH) was native protein from rat pheochromocytoma. Specificity was verified by immunoblotting from PC12 cells, as these antibodies revealed a single ~60 kDa band of the appropriate size for TH (Haycock and Waymire, 1982). To create the polyclonal antibodies to melanopsin, a synthetic peptide consisting of the 15 N-terminal amino acids of mouse melanopsin had been used as the immunogen. Specificity was confirmed by lack of staining in both retinas from melanopsin knockout mice and sections from control mice after preadsorption with the synthetic peptide (Panda et al., 2002). Melanopsin-positive and TH-positive cells identified with these antibodies in retinal wholemounts were comparable in distribution and morphology to those described in previous reports for the population of MRGCs (Dumitrescu et al., 2009; Berson et al., 2010) and DACs (Versaux-Botteri et al., 1984; Wulle and Schnitzer, 1989; Masland et al., 1993; Whitney et al., 2009) in the mouse retina.

Whole free-floating retinas were incubated in 5% normal donkey serum in PBS for 3 hrs, then rinsed with cold PBS and placed in a cocktail of primary antibodies diluted in PBS and left for 3 nights. Retinas were rinsed again in PBS and then transferred to a solution of donkey anti-rabbit secondary IgG conjugated to AlexaFluor488 (1:200; Invitrogen, catalog #A21206) plus donkey anti-sheep secondary IgG conjugated to cyanine3 (1:200; Jackson ImmunoResearch, catalog #713-165-147) diluted in PBS and left overnight. All incubation solutions contained 1% TritonX-100 and these steps were performed at 4°C. After a final set of rinses, retinas were mounted flat on clean slides using Fluoro-Gel (Electron Microscopy Sciences, catalog #17985-10).

Microscopy

Slides were transferred to an Olympus BH2 microscope equipped with epifluorescence and a video camera that was connected to a computer running BIOQUANT Nova software (BIOQUANT Image Analysis Corporation; Nashville, TN). Each retina was outlined and the positions of each MRGC and DAC were plotted and their X,Y coordinates were recorded, including the minority of MRGCs positioned in the INL. Select retinas were imaged using an Olympus Fluoview1000 laser scanning confocal microscope equipped with a motorized stage to capture overlapping image stacks using a 40× objective across the entire retina. These images were then projected and stitched together using a recently developed software program, Imago v1.0 (Mayachitra, Inc; Santa Barbara, CA), to create high-resolution whole retina mosaics. Portions of these are shown in figures 1 and 5; high resolution images of entire retinas are available as supplementary figures 1 and 2. Maximum projection images of the inner plexiform layer were created from confocal z-stacks using Metamorph (Molecular Devices; Sunnyvale, CA). All micrographs were contrast-enhanced by using the 'levels' tool in Photoshop CS3 (Adobe Systems; San Jose, CA).

Sampling

In sampling the retinas, our intention was to define a protocol that would enable sampling that should reveal both the pervasiveness and intensity of cellular clustering, where it was present. Choosing small fields that enclose cellular clusters is an effective means for showing such clustering, but it does not reveal much about how widespread the phenotype is (either locally, or across the retina), nor providing any objective assessment of the degree of clustering between different genotypes. We consequently sampled four large 1mm × 1mm fields (this sample size being the largest field that we could consistently position in a retinal quadrant), these sample sites being identified by the outline of the retina and without reference to the patterns of cellular distribution contained within the fields. (In one exception, a *Dscam*^{+/−} retina, we could not obtain uninterrupted fields this size due to the placement of the radial cuts in the retina, and so this retina provided data only on total cell number). As average retinal area was 14.8 sq. mm across the 25 retinas used in the present study, this protocol amounts a sampling of 27%, on average, of the entire retinal surface for the spatial analyses described below.

Spatial Analysis

The 2D collection of points in each sampled field was then analyzed using customized Matlab software to examine the Voronoi tessellation of the field, or using specialty software in R (Rowlingson and Diggle, 1993) to conduct the L function analysis. The Voronoi domain was calculated for each cell (excluding those cells with Voronoi domains intersecting the border of the field), and the coefficient of variation (CoV) for their Voronoi domain areas within each sample field was computed. Cellular clustering produces variability in domain area sizes, and so the CoV provides a relative measure of the intensity of this feature. The L function of each field was separately determined (see Results), as described in detail elsewhere (Raven et al., 2003). For each field, the L function was compared to those for 99 Monte Carlo simulations of random distributions matched for density. This analysis provides an assessment of whether a field differs significantly from complete spatial randomness, either because the cells space themselves apart, or because they show clustering. The latter random simulations were constrained by soma size in order to simulate the effect of soma diameter upon restricting proximity between neighboring cells. Average soma diameters (being $12.44 \pm 1.75 \mu\text{m}$ s.d., and $12.79 \pm 0.93 \mu\text{m}$ s.d., for MRGCs and DACs respectively) were determined by measuring the areas of at least 150 cell bodies of each cell type and calculating the diameters for circles of the same area. Measurements of soma size were taken from the high-resolution whole retinal mosaic of a wildtype retina. Because the clustering of MRGCs in the *Dscam*^{−/−} retinas occasionally led

to somata partially overlying one another, we also replicated the L function analysis with Monte Carlo testing using no soma size constraint; this, however, made no difference to the classification of each field as being either clustered or random, and so we report only the L function analysis in which the random simulations were constrained by soma size. This L function analysis, therefore, provides an appreciation of the pervasiveness of such a clustered phenotype, shown by the proportion of fields showing clustering.

Quantitative PCR

Once all retinas were collected, samples were processed and run simultaneously as described previously (Whitney et al., 2009). Briefly, RNA was extracted from each set of retinas (RNeasy Plus Mini Kit, Qiagen, catalog #74134) and then assessed for proper concentration, purity, and integrity (Nanodrop1000, Thermo Scientific; 2100 Bioanalyzer, Agilent). Single strand cDNA was synthesized (iScript cDNA Synthesis Kit, Bio-Rad, catalog #170-8891) and quantitative real-time PCR was run using a MyiQ Single Color Real-Time PCR Detection System (Bio-Rad). Quantification of RNA was corrected for product size, melt temperature, and PCR efficiency, while three internal control genes were used to normalize the amount of *Bax* and *Dscam* expression for each sample (as described in Vandesompele et al., 2002). A summary of all primers used in this study can be found in Table 2.

Statistics

One-way analysis of variance and post-hoc Scheffe tests were performed to identify significant differences ($p < 0.05$) in total cell number and in the CoV. L functions were tested for deviations from randomness by computing a U score for each L function and its 99 random simulations following previous approaches (Eglen et al., 2003, 2005). The U score for an L function is the integrated distance between the L function and the mean of the other 99 L functions. If the U score for the observed L function had a rank of greater than 95 out of 100, then formally we can say that the observed spatial pattern is significantly different from a completely random spatial pattern at a significance level of < 0.05 . Once deviation from complete spatial randomness has been confirmed, the pattern can then be classified as clustered (or spaced) if the L function from the observed pattern rises above (or below) the envelope of the 99 random simulations.

Results

Melanopsin Retinal Ganglion Cells

Figure 1a-d shows large representative fields from wildtype, *Dscam*^{+/-}, *Bax*^{-/-} and *Dscam*^{-/-} retinas, respectively, that had been stained to reveal the distribution of MRGCs. (High resolution figures of entire retinas are also provided on-line, as supplementary figure 1). Figure 1a shows the apparently random distribution of MRGC somata, with fine dendritic processes intermingled throughout. These features are characteristic across the entire retina (supplementary figure 1a). Optic axons are also visible coursing radially toward the optic nerve head, positioned to the right of the figure. Figure 1d, by contrast, shows the striking alterations present in the *Dscam*-mutant (*Dscam*^{-/-}) retina described by Fuerst and co-workers (2009): somata show a conspicuous clustering, while the dendritic processes of these cells are heavily fasciculated. These features are present in all four quadrants of the *Dscam*^{-/-} retina, though they vary in intensity, and are least pronounced surrounding the optic nerve head (supplementary figure 1d). Mice that are heterozygous for this *Dscam* mutation (*Dscam*^{+/-} mice) present a milder version of these phenotypic features observed in the *Dscam*^{-/-} retina (figure 1b), though showing them mainly within the peripheral parts of the retina, and not equally in all four quadrants (supplementary figure 1b). Finally, *Bax* knockout mice (*Bax*^{-/-}) show similar clustering and fasciculation features, though intermediate to those in the *Dscam*^{-/-} and *Dscam*^{+/-} retinas (figure 1c). Curiously, despite

the lack of *Bax* throughout the retina, the prevalence of these phenotypes is also restricted to the more peripheral parts of the retina, as in the *Dscam*^{+/-} retina, and to a variable extent around the retinal circumference (supplementary figure 1c). Clustering and fasciculation are not apparent at any location within the wildtype retina (supplementary figure 1a).

To determine how these phenotypic features may relate to any change in cell number, we determined the size of the entire MRGC population from all four genotypes. The average number of MRGCs per retina varied significantly between each of the four genotypes (figure 2a), showing a five-fold increase in *Dscam*^{-/-} retinas (7263 ± 595 cells, mean and standard error, and hereafter) when compared to wildtype control retinas (1340 ± 66 cells). This population was also increased in *Bax*^{-/-} retinas, exhibiting a four-fold increase (5061 ± 263 cells). A nearly two-fold increase was also found in *Dscam*^{+/-} retinas (2462 ± 232 cells), indicating a *Dscam* gene dosage effect upon both cell number and the clustering and fasciculation phenotypes, the latter of which has also recently been reported (Blank et al., 2011). By comparing the size of each population (figure 2a) with the qualitative features shown in the whole retina images (figure 1 and supplementary figure 1), the three phenotypes (cell number, clustering and fasciculation) apparently correlate with each other, each being most pronounced in the *Dscam*^{-/-} retina, all somewhat reduced in the *Bax*^{-/-} retina, and all reduced further in the *Dscam*^{+/-} retina. In summary, the manipulation of deleting *Bax* gene function yields an increase in cell number and the presence of cellular clustering and fasciculation that appears qualitatively similar to that found in *Dscam* deficient retinas.

Because the fasciculation in these retinas obscures the identification of single somata in such low-magnification images, we analysed the distribution of MRGCs by plotting the Voronoi domain of each cell across the retina (figure 2d-g). The Voronoi domain of a cell is the spatial territory closer to that cell than to any of its neighbors, and so the tessellation of the entire retina parcels the retinal area accordingly. The distribution of Voronoi domain sizes, like the distribution of somata, shows the progressive clustering phenotype as a function of increases in the size of the entire population across these four genotypes. These cells do not simply maintain their spatial relationships as density increases, for if they did, the variation in Voronoi domain areal size would be maintained. To quantify this fact, we determined the coefficient of variation (CoV; the standard deviation divided by the mean) for Voronoi domain size in these MRGC mosaics in each group of mice. Figure 2b plots the CoV for each of four 1mm × 1mm fields from every sampled retina (with the exception of one *Dscam*^{+/-} retina), showing an increase in this variability that parallels the increase in cell density (figure 2a) across these four genotypes, each being significantly different from all others. These results suggest that clustering and fasciculation are directly related to the variation in cellular density across these four genotypes, and indeed, a plotting of their CoVs as a function of density suggests a strong correlation ($r^2 = 0.65$). If, however, the relationship is considered for each genotype separately, there is little indication of a correlation between the variability of Voronoi domain size and cell density in the individual fields (figure 2c).

We also determined the L function for these same large sample fields, being the cumulative frequency distribution of distances from each cell to every neighboring cell out to a set distance (Diggle, 2002), in this case, 100 μm (similar results were obtained using an upper bound of 200 μm). The L function for a random distribution of cells climbs as a diagonal line from the origin of the plot (Diggle, 2002): for a given distance d we would expect $L(d)=d$. For a cell-type that minimizes proximity to other neighboring cells, the L function veers beneath that for a random distribution of cells ($L(d) < d$), as there are fewer shorter distances between a cell and any neighboring cell, while a cell-type that exhibits clustering

contains an excess of such shorter distances between neighboring cells, and consequently the L function veers above that for a random distribution of cells ($L(d) > d$).

Figure 3 (a-d) plots example fields for the four different genotypes, along with random distributions of cells matched in density and simulating the distribution of soma sizes found in the real data to mimic the constraint of the physical size of the soma upon proximity (e-h). Figure 3 (i-l) plots the L function for the real data shown in the fields on the left (red broken line in i-l), relative to the envelope of 99 random simulations (only one of which is shown in e-h), indicated by the grey lines. (The width of the envelopes decreases as a function of increasing density observed across the four genotypes). Importantly, the L function derived from the real data falls within the envelope of the 99 simulations for the wildtype retina (figure 3i), indicating that, for this particular field, it is not statistically discriminable from a random distribution of cells of the same density. Note, though, that the other fields shown in figure 3b-d (for *Dscam*^{+/-}, *Bax*^{-/-} and *Dscam*^{-/-}, respectively), are all significantly different from random distributions using such Monte Carlo testing (figure 3j-l). Indeed, the insets above these L functions rank-order the U score of each of these 100 L functions (Eglen et al., 2003b; 2005), this score being the difference of each L function from the mean of the other 99 L functions (shown as the central grey line within each envelope), as described by Diggle (1979). For this U score statistic, the lower the rank, the better the real data are fit by the random simulations. Using this Monte Carlo testing for each of the sampled fields, the histograms in figure 3 (m-p) show that 100% of the sampled fields derived from the *Dscam*^{-/-} retinas were clustered, 94% of the fields in the *Bax*^{-/-} retinas were clustered, 71% of the fields in the *Dscam*^{+/-} retina were clustered, while 8% of the fields from wildtype retina were clustered. That latter observation of a few wildtype clustered fields demonstrates the sensitivity of this approach for detecting deviations from random; the majority (78%) of wildtype fields were all indiscriminable from random distributions (figure 3m). None of the L functions for any of the distributions of MRGCs in each of the three experimental conditions showed evidence for homotypic avoidance (figure 3n-p), although a few of the wildtype fields did (15%; figure 3m). The relative absence of homotypic avoidance in the wildtype retinas may be because this cell-type does not typically engage in such minimal spacing behavior; more likely, it reflects the fact that there are multiple sub-types of MRGCs labelled with this melanopsin antibody, each of which may self-space independent of one another (Dumitrescu et al., 2009; Fuerst et al., 2009; Berson et al., 2010; Schmidt and Kofuji, 2011).

MRGCs can be discriminated according to their morphology (Berson et al., 2010), their depth of dendritic stratification in the IPL (Ecker et al., 2010), and their central terminations (see Schmidt et al., 2011, for review), but they are not yet discriminable as separate populations using antibodies, and the present analysis did not attempt this classification. The dendrites of the M1 and the bistratified M2 subtypes of MRGC, however, stratify in the outer (S1) stratum of the IPL, while the other subtypes (including the M2) stratify in the inner (S5) stratum. Figure 4 shows the distribution of dendritic processes within these two strata of the inner plexiform layer (left and middle columns, respectively) in example fields from each group. (Note that not all somata in the GCL could be excluded in the images on the left). These images show that aberrant fasciculation is present in both strata in all but the wildtype retina, and convey the variability of this phenotype. In conjunction with the whole retinal images shown in figure 1 and supplementary figure 1, which are Z stack projections including the entire inner retina (ganglion cell layer, inner plexiform layer, inner nuclear layer), it would appear that, like the clustering, the fasciculation is greatest in the *Dscam*^{-/-} retina and progressively reduced in the *Bax*^{-/-} and *Dscam*^{+/-} retinas.

Dopaminergic Amacrine Cells

In the wildtype retina, a single population of dopaminergic amacrine cells (DACs) is distributed across the innermost portion of the inner nuclear layer (figure 5a; supplementary figure 2a) giving rise to a fine distribution of dendrites and long axonal processes in the outermost stratum (S1) of the inner plexiform layer, as previously reported (Raven et al., 2003; Keeley and Reese, 2010). DACs in the *Dscam*^{-/-} retina show a marked fasciculation of their processes relative to wildtype retinas (figure 5d; supplementary figure 2d), as previously described (Fuerst et al., 2008). Yet, when compared with the MRGCs (figure 1d), the fasciculation and cellular clustering were still relatively modest. The *Dscam*^{+/-} retina was barely discriminable by eye from wildtype retinas (figure 5b; supplementary figure 2b), while the *Bax*^{-/-} retina showed features associated with the pattern of fasciculation (figure 5c; supplementary figure 2c) better appreciated at higher magnification, described below.

As with the MRGCs, so the DACs in *Dscam*^{-/-} retinas were numerically increased relative to wildtype retinas (1060 ± 186 cells versus 607 ± 20 cells, respectively; figure 6a). *Dscam*^{+/-} retinas, in comparison, showed only a modest increase (823 ± 53 cells). By contrast, the DAC population in the *Bax*^{-/-} retina underwent a greater than five-fold increase relative to wildtype retinas (3211 ± 302 cells). The only significant differences present were between the *Bax*^{-/-} retina and all others (figure 6a), in part because of the greater variance within the *Dscam*^{-/-} mice. These numerical results approximate the magnitudes previously reported in the increase in the size of the DAC population for both the *Dscam*^{-/-} and *Bax*^{-/-} retinas (Fuerst et al., 2008; Whitney et al., 2009).

As in the population of MRGCs, the spacing of DACs appeared to become more variable as cell density increased across these four genotypes (figure 6d-g). The CoV derived from Voronoi domain analysis confirmed this significantly increased variability in intercellular spacing for *Dscam*^{-/-} and *Bax*^{-/-} retinas relative to wildtype and *Dscam*^{+/-} retinas (figure 6b), although it did not differ between wildtype and *Dscam*^{+/-} retinas, nor between *Dscam*^{-/-} and *Bax*^{-/-} retinas despite the large difference in cell number (figure 6a). Here too, the trend between increasing cell density and CoV across all sample fields ($r^2 = 0.34$) was absent when considering any of the genotypes separately (figure 6c).

As described above for the MRGCs, we also determined the L function for each of the same sampled fields labelled for DACs. Figure 7a-d shows characteristic fields from each of the four genotypes, while figure 7e-h shows density-matched, soma-size constrained, random distributions of cells. The field in figure 7a is characteristic of DAC mosaics in wildtype retinas, showing an apparently random distribution but for the fact that cells are rarely positioned in close proximity, exhibiting an exclusion zone with a radius of about 80-100 μm (Raven et al., 2003). This deviation from randomness is borne out by L function analysis (figure 7i) showing that this single field (in a) falls well outside the envelope of 99 random simulations (note the L function is zero until about 60 μm before rising). Figure 7m indicates that 69% of all of the wildtype fields were significantly different from random, showing this tendency to minimize proximity with homotypic cells; all of the other fields could not be discriminated from random simulations, none of them showing evidence for clustering. The *Dscam*^{+/-} retinas showed fewer fields exhibiting such homotypic avoidance (39%; figure 9n), while most fields did not differ from random simulations (61%; example illustrated in figure 7j). The *Bax*^{-/-} and *Dscam*^{-/-} retinas, in comparison, never showed evidence of homotypic avoidance (figure 7o, p; respectively), and evidenced clustering with increasing frequency (50% and 67%, respectively) (figure 7k, l, o, p).

Taken together, these results indicate that DAC somata in the *Bax*^{-/-} and *Dscam*^{-/-} retinas are found in closer proximity to homotypic neighbors more often than in control retinas, and critically, more often than in density-matched random simulations; this clustering, however,

is not as severe as that for the population of MRGCs in these same retinas. This is indicated in the L function analysis by the relatively greater deviations of the real data from the random simulations for the MRGCs, when compared with the deviation for the DACs (compare figures 3k,l with 7k,l). A direct comparison of figures 1 and 5 make clear this poignant distinction between the clustering phenotypes present in these two cell types in the *Bax* or *Dscam* deficient retinas: DAC distributions show close (side-by-side) pairs of cells that occur with a frequency greater than random simulations would predict, and thereby contribute to an L function that is significantly different from random (see also Raven et al., 2003). MRGCs, by contrast, aggregate as multi-cellular clusters.

Despite the meager clustering present in the mosaic of DACs, these cells exhibit a striking fasciculation of their processes in the *Dscam*^{-/-} retina (figure 4l; 5d) that is not present in wildtype or *Dscam*^{+/-} retinas (figures 4i, 5a; 4j, 5b). Interestingly, *Bax*^{-/-} retinas also contained regions where bundles of DAC processes could be identified, yet their appearance was distinct from that seen in the *Dscam*^{-/-} retina. Specifically, the fasciculated bundles appeared less tightly packed by comparison with the *Dscam*^{-/-} retina, and there remained a dense and non-fasciculated meshwork of processes throughout the retina (figures 4k, 5c). The fascicles in the *Dscam*^{-/-} retina appeared more tightly fasciculated (figure 4l), and the retinas lacked the network of non-fasciculated processes so dominant in the other three genotypes (compare figure 5d with 5a-c). The increasing density of this network across figures 5a-c correlates with the increase in cell density; its apparent absence in the *Dscam*^{-/-} retina suggests an entirely unique feature unrelated to cell density or clustering. Whether this distinction in the *Dscam*^{-/-} retina reflects a difference in the behavior of axonal versus dendritic processes relative to the *Bax*^{-/-} retina, is currently unclear. Previous analysis of single DAC morphology in the *Bax*^{-/-} retina revealed that the dendritic processes appears to be unperturbed in their patterning, if mildly stunted in length (Keeley and Reese, 2010), suggesting that the fasciculation seen in these immunostained retinas might be associated with the bundling of axonal processes instead, although it is possible that none of the cells labelled in that study included cells participating in such fasciculation. What is clear, however, is the fact that DACs do not show quantifiable differences in clustering between the genotypes that parallel differences in cell number, unlike the MRGCs, and exhibit qualitative differences in fasciculation that do not relate, in an obvious way, to differences in cell number.

Finally, we confirmed with qPCR that *Dscam* expression is progressively reduced in the two *Dscam*-mutant mice (figure 8a, top), as expected (Fuerst et al. 2008), but that *Bax* expression is unaffected in these mice (figure 8b, top). Likewise, *Bax* expression is completely abolished in the *Bax*^{-/-} retina (figure 8b, bottom), yet there is no effect upon *Dscam* expression in these *Bax*^{-/-} mice (figure 8a, bottom).

Discussion

The present results demonstrate that deletion of *Bax* gene function produces changes in the spatial distributions of MRGCs and DACs and their processes that are, at least superficially, similar to those observed following disruption of *Dscam* gene function. In each case, the somata of individual cells are positioned closer together than in control retinas and closer than would be expected in random distributions of similarly sized cells at comparable density; additionally, their processes show a tendency to fasciculate with one another rather than extending as though impervious to the presence of homotypic neighbors, as has been demonstrated for DACs in the wildtype mouse retina (Keeley and Reese, 2010). *Dscam* function in the mouse retina is believed to mask adhesive interactions between homotypic cells, since the somal clustering and fasciculation of processes in the *Dscam*^{-/-} retina occurs primarily between cells of the same type (Fuerst et al., 2009). *Bax* gene function, by

contrast, is to promote apoptosis during normal retinal development (Ogilvie et al., 1998; Péquignot et al., 2003). Like *Bax* knockout mice, however, the *Dscam*-mutant retina contains an increase in the total number of each of these two types of cell. Why disrupting *Bax* gene function should alter cell spacing and fasciculation, while disruption of *Dscam* gene function should yield increases in cell number, each remain unclear, though ongoing developmental studies of each knockout mouse should shed further light on these questions. The following points can be gleaned from the present analysis.

First, it seems unlikely that disrupting either of these two genes is having its effect by modulating the function of the other directly. qPCR confirmed no change in *Bax* expression in the *Dscam*^{-/-} retina, and likewise no change in *Dscam*-expression in the *Bax*^{-/-} retina. *Dscam* and *Bax* are believed to govern intercellular recognition versus cell survival, respectively, and there is no established basis for a direct interaction between these pathways. Furthermore, the profile of effects is different between the two cell types, *Bax* deletion producing the greatest number of DACs and *Dscam* deletion producing the greatest number of MRGCs; clearly, these profiles cannot be explained by hypothesizing, for instance, reduced function of *Bax* in the *Dscam*-mutant retina, or vice versa. Recent studies have, however, shown that *Bax* may also mediate other non-apoptotic functions via caspase activation, including long-term depression of synaptic transmission (Jiao and Li, 2011), while disrupted *Dscam* function, impacting cellular distribution and differentiation, might be expected to alter synaptic connectivity (Millard et al., 2010) and ultimately apoptosis. Additionally, there are other recently documented examples of how *Dscam* may interact heterophilically, serving as a receptor for the axonal guidance signals Netrin and Draxin (Ly et al., 2008; Liu et al., 2009; Ahmed et al., 2011). Such unconventional mechanisms may ultimately prove to be the means by which *Dscam* and *Bax* act in a cell-type specific manner to contribute the diversity of effects reported herein.

Second, the clustering and fasciculation phenotypes appear to parallel loosely the magnitude of the changes in cell number between the genotypes for the MRGCs, suggesting that these phenotypes might simply be secondary to the changes in cell number. Yet a consideration of the CoV as a function of density within individual genotypes shows minimal correlation (figure 2c), suggesting that the overall trend is a consequence of other differences between the genotypes affecting spatial positioning independent of density. DACs, by contrast, fail to show a clear difference in CoV between genotypes with large differences in density (figure 6c), and the fasciculation phenotype was most conspicuous in the *Dscam*^{-/-} retina despite its having only a third of the number of cells present in the *Bax*^{-/-} retina. Even the L function analysis suggests more pervasive clustering in the *Dscam*^{-/-} retina relative to the *Bax*^{-/-} retina.

Third, the gene dosage effect demonstrated by the *Dscam*^{+/-} retina may be suggestive of a threshold effect, different for individual types of cells. An effect upon DACs was not detected, while MRGCs showed lesser versions of the *Dscam*^{-/-} phenotype for all three features: cell number, clustering and fasciculation. If the normal function of *Dscam* is to mask adhesive interactions between homotypic neighbors (Fuerst and Burgess, 2009), perhaps by acting as a co-receptor nullifying adhesion, then lowering the amount of *Dscam* (in the *Dscam*-heterozygote) or increasing the frequency of homotypic interactions (in the *Bax* knockout) may each manage to overcome this adhesive masking between like-type processes.

Why these mosaics exhibit cellular clustering remains a mystery. Initial reports of the DAC mosaic in *Dscam*^{-/-} retinas suggested that these cells acquire their clustering phenotype secondary to the fasciculation of their processes, and the same has been suggested for the MRGCs, as if these cells are pulled across the retina by the adhesive forces between

fasciculating processes (Fuerst et al., 2008; 2009). An alternative interpretation is that these mature distributions are sculpted through selective cell loss during development, although this explanation has usually been put forward as a means to increase the regularity in the patterning of retinal mosaics (Eglen, 2006). While other studies have shown that naturally occurring cell death participates in eliminating close DAC neighbors (Raven et al., 2003), it is difficult to conceive of the clustered distribution of MRGCs in the *Dscam*^{-/-} retina arising simply from a lack of spatially-selective cell death. Interestingly, the clustered distribution of the MRGCs is similar to one recently described in mice deficient in the transcription factor gene *Barhl2* (Ding et al., 2009): those retinas show a clustered phenotype of cholinergic amacrine cells coupled with a two-fold increase in cell number, yet this population of cells does not express *Dscam* nor *Dscam1* (Fuerst et al., 2008; 2009), and does not undergo Bax-mediated cell death during development (Whitney et al., 2008). Furthermore, this clustering is unique to the mosaic of cholinergic amacrine cells in the ganglion cell layer, and is not observed within the mosaic of cells in the INL despite these exhibiting a similar increase in number (Ding et al., 2009).

The present results then, in conjunction with those discussed above, would suggest that there are multiple means of producing a clustered and fasciculated phenotype. Having functional *Dscam* is no guarantee of a cell's personal space nor disregard for its homotypic neighbors, made clear by the *Bax*^{-/-} phenotype. By contrast with the MRGCs and DACs, many other retinal cell-types appear to be acutely responsive to the presence of homotypic neighbors in the wildtype retina, distributing their processes evenly to yield a constant coverage of the retinal surface (Reese, 2008). The DAC and MRGC types appear to be exceptions to that rule, participating as they do in the non-image forming circuits of the visual pathway (Viney et al., 2007; Vugler et al., 2007; Zhang et al., 2008). It will be interesting to compare the same phenotypic features for other retinal cell types across a range of genotypes to understand better the normal role of *Dscam* in the formation of intercellular spacing and dendritic coverage, and to appreciate more fully the extent to which these phenotypic features in the *Dscam*^{-/-} and *Bax*^{-/-} retinas diverge, potentially lending insight into their formation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Large fields of retinal wholemounts that had been immunofluorescently labelled using antibodies to melanopsin to reveal the population of MRGCs. a: Wildtype retina showing the normal distribution of somata and processes. b: *Dscam*^{+/-} retina showing several clusters of somata and associated fasciculation of their dendrites. c: *Bax*^{-/-} retina showing an increased frequency of clustering and fasciculation. d: *Dscam*^{-/-} retina displaying the severe and widespread clustering and fasciculation phenotype. Scale bar = 500 μ m. High resolution images of entire retinas from each of these four genotypes are provided in supplementary figure 1, showing the regional variability in these phenotypic features across the conditions.

Figure 2.

a: Total number of MRGCs counted across the entire retina from different mice in control, *Dscam*^{+/-}, *Bax*^{-/-} and *Dscam*^{-/-} mice. N = the number of retinas sampled. b: Coefficient of variation (CoV) for the Voronoi domain areas in MRGC mosaics from control, *Dscam*^{+/-}, *Bax*^{-/-} and *Dscam*^{-/-} retinas. N = the number of sampled fields. Means and standard error are plotted. Asterisks indicate significant differences from all other genotypes. c: Scatterplots showing the relationship between the CoV and cell density for the individual fields from figure 2b. d-g: Example plots of MRGC position and Voronoi tessellation from control (d), *Dscam*^{+/-} (e), *Bax*^{-/-} (f), and *Dscam*^{-/-} (g) retinas.

Figure 3.

L function analysis of MRGCs from control (a, e, i, m), *Dscam*^{+/-} (b, f, j, n), *Bax*^{-/-} (c, g, k, o), and *Dscam*^{-/-} (d, h, l, p) retinas. a-d: 1mm × 1mm sample fields of MRGCs. e-h: Random simulations of fields constrained by cell size and matched in density to those shown in a-d. i-l: L functions derived from the real fields shown in a-d (red broken lines) relative to the envelope of L functions from 99 random simulations like those in e-h (continuous grey lines). Red broken lines extending above the envelope indicate distributions that show clustering, while red broken lines extending below the envelope indicate distributions that show homotypic avoidance (spacing). A U score was calculated for each of the 100 L functions, describing how much each L function differed from the average of the other 99 L functions (grey line within the envelopes), shown in the horizontal plots within the insets. m-p: Histograms display the percent of fields that were significantly different from random simulations, showing evidence of clustering or spacing according to the L function.

Figure 4.

a-h: Micrographs showing the melanopsin-positive dendrites in the inner plexiform layer for control (a, e), *Dscam*^{+/-} (b, f), *Bax*^{-/-} (c, g), and *Dscam*^{-/-} (d, h) retinas, separating those processes that stratify in stratum (S) 5, adjacent to the ganglion cell layer (a-d) from those that stratify in S1, adjacent to the inner nuclear layer (e-h). Notice that the fasciculation of MRGC dendrites occurs in both strata of the IPL in the *Dscam*^{+/-}, *Bax*^{-/-} and *Dscam*^{-/-} retinas. i-l: The distribution of TH-positive processes in S1 of the inner plexiform layer is also shown in control (i), *Dscam*^{+/-} (j), *Bax*^{-/-} (k) and *Dscam*^{-/-} (l) retinas. Fasciculation was apparent in portions of both the *Bax*^{-/-} and *Dscam*^{-/-} retinas only, but was distinguishable by the looser nature of the fasciculation in *Bax*^{-/-} and by the relative absence of a network of non-fasciculated processes in the *Dscam*^{-/-} retina. Scale bar = 100 μ m.

Figure 5.

Large fields of whole retinas immunolabelled using antibodies to tyrosine hydroxylase to reveal the population of DACs. a: Wildtype retina showing the normal distribution of somata and processes. b: *Dscam*^{+/-} retina showing somewhat greater variability in spacing between somata but a relatively normal distribution of processes. c: *Bax*^{-/-} retina showing elevated density of DACs that are irregularly spaced, with limited fasciculation of processes. d: *Dscam*^{-/-} retina displaying comparably irregular spacing between DAC somata in the presence of a conspicuous fasciculation of their processes. Scale bar = 500 μ m. High resolution images of entire retinas from each of these genotypes are provided in supplementary figure 2.

Figure 6.

a: Total number of DACs counted across the entire retina from different mice in control, *Dscam*^{+/-}, *Bax*^{-/-} and *Dscam*^{-/-} mice. N = the number of retinas sampled. b: Coefficient of variation for DAC mosaics from control, *Dscam*^{+/-}, *Bax*^{-/-} and *Dscam*^{-/-} retinas. N = the number of sampled fields. Means and standard error are plotted. Asterisks indicate significant differences from all other genotypes in a, or indicate specific differences in b. c: Scatterplots showing the relationship between the CoV and cell density for the individual fields from figure 6b. d-g: Example plots of DAC position and Voronoi tessellation from control (d), *Dscam*^{+/-} (e), *Bax*^{-/-} (f), and *Dscam*^{-/-} (g) retinas.

Figure 7.

L function analysis of DACs from control (a, e, i, m), *Dscam*^{+/-} (b, f, j, n), *Bax*^{-/-} (c, g, k, o), and *Dscam*^{-/-} (d, h, l, p) retinas. Conventions as for figure 3. Unlike the MRGCs, many of the L functions for the fields of DACs in the wildtype and *Dscam*^{+/-} retinas showed evidence of homotypic avoidance (spaced), while none showed evidence of being spaced in the *Bax*^{-/-} and *Dscam*^{-/-} retinas. L function analysis detected clustering only in the *Bax*^{-/-} and *Dscam*^{-/-} retinas.

Figure 8. qPCR analysis for *Dscam* and *Bax* expression. *Dscam* expression was progressively reduced in the *Dscam*^{+/-} and *Dscam*^{-/-} retinas (a, top histogram), as previously described for this loss-of-function *Dscam* allele (Fuerst et al., 2008). *Bax* expression, by contrast, was unaffected in the *Dscam*-mutant retinas (b, top histogram). *Bax* expression was completely abolished in the *Bax*^{-/-} retina (b, bottom histogram), while *Dscam* expression was unaffected (a, bottom histogram). Transcript levels were normalized to the mean wildtype expression for each comparison. N = the number of retinas in each group.

Table 1

Primary antibodies used in the study

Antigen	Immunogen	Manufacturer	Dilution
Tyrosine Hydroxylase	Native protein from rat pheochromocytoma (Haycock and Waymire, 1982)	Millipore (Billerica, MA); sheep polyclonal; #AB1542	1:10,000
Melanopsin	Synthetic peptide consisting of 15 N-terminal amino acids of mouse melanopsin (Panda et al., 2002)	Gift from Dr. Ignacio Provencio, University of Virginia; rabbit polyclonal	1:10,000

Table 2

Primers used for quantitative PCR.

Gene	Primers (5' to 3')	Product Length	Melt Temperature	Calculated Efficiency
Down Syndrome Cell Adhesion Molecule	S: ACGTAAGAGGGCCTGCAAGC A: CACCTGGCGGTGGTTGAAAG	156bp	82°C	1.80
Bcl2 Associated Protein-X	S: AGATGAACTGGACAGCAATATGG A: GCAAAGTAGAAGAGGGCAACC	150bp	82°C	1.81
Glyceraldehyde 3-Phosphate Dehydrogenase	S: AATGTGTCCGTCGTGGATCTGA A: AGTGTAGCCCAAGATGCCCTTC	117bp	82°C	1.94
Beta-2 Microglobulin	S: GGAGAATGGGAAGCCGAACATAC A: AGAAAGACCAGTCCTTGCTGAAG	143bp	82°C	1.85
TATA-Binding Protein	S: GTTG GTGATTGTTGGTTTAAGGG A: GGAAGGCGGAATGTATCTGG	197bp	76°C	1.84