Published in final edited form as:

J Comp Neurol. 2008 May 1; 508(1): 1–12.

Spatial Patterning of Cholinergic Amacrine Cells in the Mouse Retina

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Abstract

The two populations of cholinergic amacrine cells in the inner nuclear layer (INL) and the ganglion cell layer (GCL) differ in their spatial organization in the mouse retina, but the basis for this difference is not understood. The present investigation has examined this issue in six strains of mice that differ in their number of cholinergic cells, addressing how the regularity, packing and spacing of these cells varies as a function of strain, layer and density. The number of cholinergic cells was lower in the GCL than in the INL in all six strains. The nearest neighbor and Voronoi domain regularity indexes as well as the packing factor were each consistently lower for the GCL. While these regularity indexes and the packing factor were largely stable across variation in density, the effective radius was inversely related to density for both the GCL and INL, being smaller and more variable in the GCL. Consequently, despite the lower densities in the GCL, neighboring cells were more likely to be positioned closer to one another than in the higher-density INL, thereby reducing regularity and packing. This difference in the spatial organization of cholinergic cells may be due to the cells in the GCL having been passively displaced by fascicles of optic axons and an expanding retinal vasculature during development. In support of this interpretation, we show such displacement of cholinergic somata relative to their dendritic stalks, and a decline in packing efficiency and regularity during postnatal development that is more severe for the GCL.

Keywords

Voronoi domain; nearest neighbor; packing factor; effective radius; regularity index; starburst amacrine cell; Bax

Introduction

Retinal nerve cells are distributed across their respective layers as regular arrays, commonly called retinal mosaics. The regularity in their distributions ensures uniformity in their contribution to visual processing at every locus upon the retinal surface, effected by their synaptic connectivity within the plexiform layers (see Reese, 2007, for review). One type of retinal neuron, the cholinergic amacrine cell, is positioned in either the ganglion cell layer (GCL) or in the inner nuclear layer (INL), giving rise to dendritic processes that stratify in narrow bands within the inner or outer parts of the inner plexiform layer (IPL), respectively (Masland & Tauchi, 1986). Their dendrites spread extensively, overlapping those of neighboring cells by a factor of 20-70, depending on the species, establishing generally circular dendritic fields that are immune to variations in local cholinergic density (Farajian et al., 2004; Keeley et al., 2007). The two populations have comparable morphologies, and are often described as “symmetrical” or “matching”, playing a role in the extraction of the direction of

motion for both the ON and OFF visual pathways within the inner and outer parts of the IPL (Yoshida et al., 2001; Euler et al., 2002; Vaney and Taylor, 2002; Lee and Zhou, 2006).

In the C57BL/6J mouse retina, the two populations have been reported to differ in their spatial organization, with those in the INL being regular while those in the GCL tiling the retina “scarcely better than a random distribution” (Galli-Resta et al., 2000). This distinction between the layers seemed puzzling, given the presumably comparable developmental events that lead to the establishment of these two populations of cells, prompting us to re-examine the nature of this difference in greater detail. Specifically, we have examined the spatial organization of the cholinergic amacrine cells in the C57BL/6J mouse retina and in the A/J mouse retina, as well as in four different recombinant inbred strains that vary in the size of their cholinergic amacrine cell population. Our intention was to understand how the regularity, packing and spacing of cholinergic amacrine cells varies as a function of layer, strain and density. We first show that the GCL population is sparser than the population present in the INL in all six strains. We confirm that the mosaic in the GCL is less regular and less efficiently packed relative to that in the INL in each strain, but demonstrate that it is reliably discriminated from random simulations. We show that the spacing of cells is inversely related to local density, but that this relationship is variably defective in the GCL. Despite the lower density in the GCL, cells are more likely to be positioned closer to one another than in the INL, contributing to the variability in this relationship. We speculate that displacement of a proportion of cells in the GCL, produced as a consequence of the delayed expansion of the retinal vasculature and the growth of axon fascicles in the GCL during the first postnatal week, contributes to the lower regularity and packing in the GCL, and we confirm that these indexes are higher in the GCL during early development. Consistent with this, we show that cholinergic amacrine cells in the GCL are frequently displaced relative to blood vessels and optic fascicles, and provide evidence that somal displacement relative to the dendritic stalk is greater for cells in the GCL. Finally, we show that the *Bax* knockout retina, containing a thicker GCL due to an excess number of retinal ganglion cells, has fewer closely-spaced cells generating a more efficiently packed mosaic. These results support the hypothesis that cholinergic amacrine cells in the GCL are displaced during normal development; such movement degrades the orderliness of the mosaic, yet should leave its functional dendritic coverage intact.

Materials and Methods

C57BL/6J (B6) and A/J (A) mice, as well as four recombinant inbred strains of the AXB/BXA strain-set (AXB4/PgnJ, AXB5/PgnJ, AXB24/PgnJ and BXA26/PgnJ), being derived from these two inbred laboratory strains, were purchased from The Jackson Laboratory. The recombinant inbred strains are homozygous at every genetic locus, but differ in the regions along each chromosome that are derived from each parental strain. Because polymorphisms in multiple genes contribute to the variation in total cell number (i.e. by regulating proliferation, fate determination and cell death), the recombinant inbred strains present phenotypes that vary between, and sometimes beyond, those present in the parental strains. These four recombinant strains were chosen to sample a range of densities spanning that normally present across the retina of the two parental strains.

Each mouse was given a lethal dose of sodium pentobarbital (120mg/kg, i.p.), and once heavily anesthetized, was perfused intracardially with 2 ml of 0.9% saline followed by 50 ml of 4% paraformaldehyde in 0.1M sodium phosphate buffer over fifteen minutes (pH = 7.2 at 20°C). All procedures were conducted under authorization by the Institutional Animal Care and Use Committee at UCSB, in accord with the ARVO Statement for the Use of Animals in Ophthalmic and Visual Research. Eyes were dissected and post-fixed for an additional fifteen minutes. Retinas were then dissected whole, rinsed in phosphate buffer and pre-incubated in normal donkey serum for 3 hours, then transferred to a choline acetyltransferase (ChAT)

antibody (Table 1) containing 1% Triton-X100 in phosphate-buffered saline (PBS), and agitated for five days at 4°C. The immunogen for the ChAT affinity-purified antibody was human placental enzyme. The antibody has been characterized extensively in mouse retinal tissue (see Kang et al., 2004; Heinze et al., 2007), and has been shown to label an expected 68-70 kD band in immunoblots (Brunelli et al., 2005). Retinas were then rinsed in PBS, and incubated in donkey anti-goat IgG conjugated to Cy2 (at 1:200; Jackson ImmunoResearch Labs; West Grove, PA) overnight. Retinas were finally rinsed in PBS followed by phosphate buffer, and mounted on clean slides under a coverslip in the same.

One retina from each mouse was examined using a 20× objective on an Olympus BHS microscope equipped for fluorescence microscopy, using a Sony video camera and X-Y stage-encoders linking the microscope to a computer running Bioquant Nova Prime software (R&M Biometrics; Nashville, TN). Individual fields were sampled from the central and peripheral retina at roughly one-third and two-thirds of the distance from the optic nerve head to the retinal circumference, in each of the four quadrants, sampling the cholinergic amacrine cell mosaic in both the GCL and the INL at each of these eight loci. Every labeled cell was identified, and its X-Y coordinate determined. A total of 31 mice were sampled, being 7 B6 retinas, 8 A retinas, 3 AXB4 retinas, 4 AXB5 retinas, 5 AXB24 retinas, and 4 BXA26 retinas, yielding 237 total sampled fields in both the INL and GCL. Sample fields were 59,220 sq. μm in area, the eight fields in a retina averaging about 3% of the total retinal area. Central and peripheral densities were averaged, and the average of those averages was calculated (occasionally, a single quadrant at either the central or peripheral field could not be sampled, leading to this averaging of the averages) and multiplied by retinal area to estimate the total number of cells in the INL and in the GCL.

For each field, the X-Y coordinates of every labeled cell were identified using Bioquant, and used to determine the nearest neighbor (NN) distance for each cell as well as Voronoi domain (VD) area for each cell, as previously described (Raven et al., 2005a). The Voronoi domain of a cell is the area of all points in the plane of the retina that are closer to that cell than to any other cell. The collection of nearest neighbor distances or Voronoi domain areas was then used to calculate a regularity index (RI), being the average NN distance or VD area divided by the standard deviation. Autocorrelation analysis was also performed, plotting the position of all cells in a field relative to every other cell. Such spatial correlograms reveal a central region surrounding the origin where local density is lower than all further distances from the origin, indicating the presence of an “exclusion zone”, indicative of a tendency for cells to avoid being positioned in close proximity to one another. An indirect measure of the size of this exclusion zone is the effective radius (ER), calculated for each field from the density recovery profile (Rodieck, 1991), revealing the minimal spacing constraints operating within a field. Packing factors (PF) were also calculated (as previously described; Raven et al., 2005a), being an index of how closely a mosaic approaches a hexagonal matrix, extending from 0, for a random distribution of points, to 1, for perfect hexagonal packing (Rodieck, 1991). Like the RI, the PF is also a scale-independent measure. For every analyzed field, a random simulation was also generated for the INL and GCL in which an identical number of cells were positioned randomly within a field of equivalent dimensions, being constrained only by preventing somas from overlapping. Simulated cells were assigned a size of 10 μm ($\pm 1 \mu\text{m}$) based upon previous measures of immunolabeled cells in the C57BL/6 retina (Farajian et al., 2004), in order to estimate the effects of soma size alone upon each of these indexes. The NNRI, VDRIs, PFs and ERs for the real biological mosaics and for the random simulations in each layer were averaged to generate strain averages, while their values for the biological fields were plotted as a function of density, for which Pearson correlation coefficients were calculated, using a p value of < 0.01 for statistical significance, all as previously described (Raven et al., 2005a).

Similar analyses were conducted on developing C57BL/6J retinas, to see if the reduced regularity and packing of the GCL mosaics emerges gradually during development. Three P5 retinas and four P3 retinas were analyzed, as above, using a size of either $8 \pm 1 \mu\text{m}$ (P5) or $7 \pm 1 \mu\text{m}$ (P3) for simulating soma size in the matching random simulations, based upon the average size of 100 labeled cells measured from both layers at each of these ages.

We also examined the *Bax* knockout mouse (obtained from The Jackson Laboratory and bred and genotyped at UCSB) using identical procedures, determining the total number of cholinergic amacrine cells in each layer, as well as the NNRI, VDRI, PF and ER for each field in both layers. A Student's *t* test was used to determine significant differences ($p < 0.05$).

In some cases, retinal sections and wholemounts from postnatal and adult B6 mice, and from adult *Bax*^{+/+} and *Bax*^{-/-} mice, were also labeled using a neurofilament antibody (Table 1). The generation of the antibody to the 150 kD neurofilament subunit and its blot specificity was described in Karlsson et al. (1989), and its staining pattern was recently illustrated in developing mouse retina (Raven et al. 2005b). Retinas were then rinsed in PBS, and incubated overnight in donkey anti-rabbit IgG conjugated to either AMCA or Cy-3 (1:200; 711-155-152 and 711-165-152, respectively; Jackson ImmunoResearch Labs; West Grove, PA).

Some tissues were also incubated overnight at 4°C in lectin PNA from *Arachis hypogaea* (peanut) conjugated to Alexa Fluor 647 (1:1000; Invitrogen, L32460; Eugene, OR) in 1% Triton-X100 in PBS, to label blood vessels, and some were additionally labeled to reveal astrocytes using a Cy3-conjugated antibody to glial fibrillary acidic protein (Table 1). Immunoblot studies confirmed the specificity of the GFAP antibody (Debus et al., 1983), while its use in the adult and developing mouse retina to label retinal astrocytes has been previously published, in retinal sections (Johnson et al., 1997) and in wholemounts (Miyawaki et al., 2004). For fluorescent Nissl counterstaining, tissues were incubated overnight at 4°C in either NeuroTrace 640/660 or 530/615 (1:100; Invitrogen; N21483 or N21482, respectively; Eugene, OR) in 1% Triton-X100 in PBS. These were examined using an Olympus Fluoview laser scanning confocal microscope, in which image stacks were collected at 1 μm intervals across 5 μm for retinal sections, or across the full thickness of the inner retina in wholemounts. Final images were contrast-enhanced and adjusted for brightness using Adobe Photoshop.

To compare the GCL mosaic to an INL mosaic that had been randomly depleted to achieve an identical density, we employed an algorithm that randomly selected 15% of the population in each INL mosaic from the A strain. These depleted mosaics were then also analyzed for their regularity, packing and spacing, as above.

We also measured the degree of displacement between single, filled, starburst amacrine cell somata in the GCL and INL in mature B6 retinas from the location of the juncture of their dendritic stalks with their primary (radiating) dendrites within the IPL. Lightly fixed retinas were immunostained for ChAT, and single labeled cells in the GCL were impaled with a micropipette filled with DiI. Cells in the INL were similarly filled but without the aid of ChAT-immunostaining, targeting cells of comparable size until a sizeable number of cholinergic cells had been labeled. The labeling, confocal microscopy and morphometric procedures have been described elsewhere (Keeley et al., 2007). Specifically, we were interested in identifying the spatial displacement (in microns) in the plane of the retina between the center of each soma in the GCL or INL with the position of the dendritic stalk where it reached the ON or OFF stratum, respectively, within the IPL.

Results

The total number of cholinergic amacrine cells in the six strains examined ranged from 27,000 to 40,000. This difference was not associated with any systematic difference in overall retinal

area (figure 1a). A greater proportion of this total complement was positioned in the INL relative to the GCL in all six strains (figure 1b), with the greatest difference being in the BXA26 strain, and the smallest difference being in the AXB5 strain, both absolutely and relative to total number. The number in the GCL extended from 12,400 in the A strain, to 17,400 in the BXA26 strain, while in the INL it ranged from 14,400 in the A strain, to 22,300 in the BXA26 strain (figure 1b).

The mosaic of cholinergic amacrine cells in the INL (figure 2b, d) appeared more regular than did the mosaic in the GCL (figure 2a, c), regardless of the strain examined. Nearest neighbor analysis confirmed this difference between the layers in every strain (figure 3a, left set of paired bars), with the average NNRI ranging from 4-5 for the INL samples, while hovering around 3 for the GCL samples. That average NNRI for the GCL mosaics was greater than the index derived from a theoretical random distribution (being 1.91; Cook, 1996), but it was no greater than random simulations constrained by cholinergic soma size (figure 3a, compare black bars on the left with dark grey bars on the right). In fact, the average NNRI for the GCL mosaics was sometimes lower than that observed for the random simulations due to the partial overlap occasionally present amongst neighboring cholinergic cells, enabling them to be closer to one another than the distance of one cell diameter (e.g. figure 2a, c).

The nearest neighbor analysis considers the relationship between a cell and only one of its neighbors, and consequently does not always discriminate real distributions of nerve cells from random distributions (Eglen et al., 2003b). This was clearly the case with cholinergic amacrine cells in the GCL, for when Voronoi domain analysis was conducted, the average VDRI for the INL was again greater than that for the GCL (figure 3b, left set of paired bars), but the VDRI for the GCL was also consistently larger than for the random simulations, like the INL mosaic (compare with right set of paired bars in figure 3b).

Another index of patterning in the mosaic, the packing factor (PF), addresses how closely a distribution of cells approximates a hexagonal matrix. We compared the PF for the mosaics in the INL and GCL to address whether this measure also discriminates the two layers from one another or from random simulations. As with the NNRI and the VDRI, the average PF was consistently higher in the INL relative to the GCL (figure 3c, left set of paired bars), but, unlike the NNRI, PF for the GCL was also reliably higher than for the random simulations (compare with right set of paired bars in figure 3c). These results make clear that the mosaic in the mouse retina is more regular and efficiently packed in the INL relative to the GCL, but both layers are readily discriminable by their regularity and their packing from random distributions of cells.

It is important to stress that the differences in the regularity and packing of the mosaics in the INL and GCL are not simply a consequence of undersampling (Cook, 1996); that is, the random deletion of 15% of the cells from an INL mosaic does not produce a mosaic with spatial properties identical to the mosaic in the GCL. For example, randomly eliminating this proportion of cells from the INL mosaic in the A strain retinas reduces the VDRI and the PF to levels approaching that found in the GCL mosaic (figure 4b, c), but hardly affects the NNRI (figure 4a). By contrast, all three measures are appreciably different between the GCL and INL in the A strain retinas, as they are for the other five strains (figure 3a-c). Those differences in the biological mosaics cannot simply reflect the effects of cell death killing off 15% of the mosaic after the positioning of all cells had been set, and nor by a failure to label the full population by this amount.

The effective radius (ER) is relatively immune to the effects of undersampling (Cook, 1996); this is borne out in the random deletion study described above, where a 15% elimination of cells from the INL does not alter the ER at all (figure 4d). In comparison, the ER for the

biological mosaics was larger for the INL than for the GCL (figure 3d, left set of paired bars). Despite the lower density of cells in the GCL, the ER in the GCL was lower, apparently due to the more frequent presence of close-neighbor pairs (e.g. figure 2). Still, the frequency of such short-distance pairings was less frequent than would be achieved in a random simulation, evidenced by the fact that the ER for the GCL mosaic was still greater than in the random simulation, i.e. where only soma size constrains proximity (compare with right set of paired bars in figure 3d). The smaller ER in the GCL in the presence of lower densities of cells, therefore, accounts for the reduced regularity and packing efficiency in this layer.

If we consider the regularity or packing of the individual fields themselves, regression analysis showed that neither of the RIs nor PF varied significantly as a function of density in the GCL (figure 5a-c, left). Largely comparable results were obtained for the INL (figure 5a-c, right). Although conspicuous variability in these measures was present, these results show that the patterning of the mosaic was largely maintained despite a nearly three-fold variation in density within either mosaic. ER must, therefore, vary inversely with density in order to preserve these other measures of regularity and packing across density, and was confirmed to do so in both the GCL and INL (figure 5d, left and right). The negative correlation was, however, nearly twice as strong for the INL than for the GCL: at any given density, the variation in ER was far greater in the GCL than it was in the INL, suggesting that some feature associated with the GCL has a variably perturbing effect upon the positioning of cells.

One possibility is that, after the mosaic is laid down following radial migration and tangential dispersion of the cholinergic amacrine cells, occurring perinatally (Galli-Resta et al., 1997; Reese et al., 1999), the growth of optic axons, the invasion of astrocytes, the expansion of the retinal vasculature (see Gariano and Gardner, 2005; Fruttiger, 2007, for reviews) and the settling of these components into the GCL causes a passive displacement of the cells therein. Retinal astrocytes, being immigrants from the optic nerve head (Ling and Stone, 1988; Watanabe and Raff, 1988), have migrated roughly half-way across the inner surface of the retina on the day of birth (Johnson et al., 1997), while retinal vessels, following this wave of astrocytic invasion, enter the eye on the day of birth and extend their vascular network to the retinal periphery during the first ten postnatal days (Gariano and Gardner, 2005). Early on, the optic fascicles (figure 6a) and blood vessels (figure 6c) lie beneath the GCL, yet as development proceeds and retinal area expands, these components of the inner-most retina come to reside closer to, or even intermingled with, the somata of the GCL (Kim et al., 2000; Zhang et al., 2005) (figure 6b, d). Indeed, wholemounts of mature retina frequently show cholinergic amacrine cells in the GCL aligned alongside blood vessels (unlabeled in figure 2a, c) and optic fascicles (labeled for neurofilaments in figure 6e; note cells indicated by arrowheads).

Cholinergic amacrine cells begin differentiating their dendritic arbors shortly following their migration, with multiple primary dendrites extending from the somata into their respective strata in the IPL (Stacy and Wong, 2003). As development proceeds and the IPL thickens, the nucleus translocates to yield a single dendritic stalk giving rise to those multiple primary radiating dendrites (Kim et al., 2000; Zhang et al., 2005). One index that these developmental events may cause a shifting of somata is provided by the displacement of the soma relative to the juncture of the dendritic stalk with those primary radiating dendrites in the IPL (figure 6f). For twenty-one mature B6 starburst amacrine cells in the GCL that had been individually labeled, the mean (and standard deviation) displacement was $7.0 \pm 2.04 \mu\text{m}$, while for twelve cells in the INL, it was $3.3 \pm 1.77 \mu\text{m}$. All but two of the cells in the INL had a displacement that was less than half the average soma size ($\approx 5 \mu\text{m}$), whereas all but three of the cells in the GCL had a displacement that was greater than this, some greater than $10 \mu\text{m}$ (figure 6g). While such somal displacements clearly differed between the cells of the two mosaics, measurements of the position of the stalk relative to the geometric center of the dendritic field did not differ for those cells in the GCL versus the INL. These somal displacements appear small, but they

are sufficient to render close pairings between neighboring cells, given average soma size ($\approx 10 \mu\text{m}$) and average nearest neighbor distance ($\approx 20 \mu\text{m}$) (Galli-Resta et al., 2000; Farajian et al., 2004). Such displacement cannot reflect directional outgrowth during earlier development; it must occur secondarily as the components of the innermost retina come to reside in the same layer of the starburst amacrine cells during postnatal development (Kim et al., 2000; Zhang et al., 2005).

Given this displacement of cells, one would expect our scale-independent measures of patterning (RI and PF) to decline as a function of development. We consequently examined this by comparing the INL and GCL mosaics at P3 and P5 with more mature retinas in the B6 strain. (Because ER will also change simply as a function of retinal growth, we have not included this comparison across ages). We first confirmed that the size of the population had achieved adult numbers by these stages (figure 7a). We then examined the mosaic properties, assessed by RI and PF, as a function of development. Both the NNRI (figure 7b) and the PF (figure 7d) showed a decline after the first postnatal week, yet the VDRI did not (figure 7c), consistent with the expectation that small displacements that occasionally bring cells closer together will not change the size of Voronoi domains as much as they will impact NN and PF statistics. The NNRI and VDRI were both greater in the INL at all stages, whereas the magnitude of the reduction in the PF as a function of development appeared greater for the GCL than for the INL.

As a final test of this hypothesis that the innermost retinal components displace single cholinergic amacrine cells, thereby perturbing mosaic patterning, we have examined the *Bax* knockout retina. *Bax*^{-/-} mice have excess numbers of retinal ganglion and amacrine cells (Mosinger Ogilvie et al., 1998; Péquignot et al., 2003), as do *Bcl-2* overexpressing transgenic mice (Martinou et al., 1994; Strettoi et al., 2002), due to the respective pro- and anti-apoptotic actions of these genes (Linden and Reese, 2006). As a consequence, the GCL and INL are thicker than in control littermates, spacing the cholinergic amacrine cells further from the optic fiber layer, astrocytes and blood vessels (figure 6h, i). Our prediction was that the mosaic of cholinergic amacrine cells would be *less* perturbed in the presence of these excess retinal ganglion cells for this reason. We first confirmed that cholinergic amacrine cell number is not affected in *Bax*^{-/-} retinas (figure 8a), as had previously been shown for the *Bcl-2* overexpressing retina (Strettoi et al., 2002), unlike other types of amacrine cells, including the dopaminergic amacrine cells, which undergo a 4- to 9-fold increase, respectively (Raven et al., 2006; Strettoi et al., 2002). Figure 8b shows two examples from central fields, in which the incidence of closer neighbor pairs is conspicuously greater in the *Bax*^{+/+} retina. Indeed, the frequency of nearest neighbor distances less than $10 \mu\text{m}$ was twice as great for the *Bax*^{+/+} samples than for the *Bax*^{-/-} samples ($n = 24$ and 23 fields, respectively). While the regularity indexes for both the nearest neighbor analysis and the Voronoi domain analysis showed a trend toward increased regularity in the *Bax*^{-/-} samples (figure 8c, d), only the VDRI approached significance. Packing efficiency (figure 8e), on the other hand, and the ER (figure 8f), were both significantly increased in the *Bax*^{-/-} retina. None of these measures showed a significant difference in the INL (not shown).

Discussion

The present study has shown that the mosaic of cholinergic amacrine cells in the INL is more regular and efficiently packed than is the mosaic in the GCL. Contrary to a previous report (Galli-Resta et al., 2000), concluding that the mosaic in the GCL “tiles the retina hardly better than a random distribution of the same number of cells”, the present study has shown that the GCL mosaic is readily discriminated from random mosaics, in six different strains of mice, across a wide range of densities. We conclude that the mosaic in the GCL is not at all like a random distribution of cells, but is, rather, a degraded version of a more regular mosaic,

exhibiting the same tendency to engage in self-spacing in association with local density (e.g. figure 5d). What, then, might account for the degradation in this organization?

The two mosaics differ in density, with that in the GCL being about 15% lower than that in the INL. The simple depletion of a regular mosaic by 15%, by a process of cell death (or by experimental error, through undersampling due to insufficient detection), will degrade the patterning of the mosaic, evidenced by RI and PF analyses. The NNRI is, however, the most forgiving at low depletions in regular mosaics, since most cells will have another remaining neighbor that is at a comparable distance (Cook, 1996), thereby preserving the regularity index. In the presence case, simulated depletions affected the VDRI and PF while leaving the NNRI relatively intact, consistent with the above. But ER is the most robust measure in the presence of undersampling (Cook, 1996), and it did not change following a 15% simulated depletion, yet it was consistently lower in the GCL of the real biological mosaics (figure 3d). We conclude that undersampling (through either the biological process of naturally occurring cell death, or by virtue of experimental error) cannot account for the difference in the patterning of the mosaics between the GCL and INL.

The fact that ER was lower in the GCL is consistent with the observation that cells in the GCL were more frequently positioned side-by-side than in the INL (e.g. figure 2). We confirmed their greater frequency by counting the proportion of cells in each mosaic in another set of adult B6 retinas at an older age (P78) that were within one soma diameter (10 μ m) of another cell, finding nearly twice as many in the GCL. Similar conclusions can be drawn from published images comparing INL and GCL mosaics in other species, particularly the rabbit (Vaney et al., 1981; Brandon, 1987). This difference, cannot, of course, be a simple consequence of greater chance occurrences of side-by-side positioning in the mouse retina, since cell density is lower in the GCL. The presence of such close neighboring pairs should lower ER, but as it occurs irregularly (e.g. figure 2), would be expected to produce this lowering of ER inconsistently, as is borne out in figure 5d (compare the variability at any density in the left panel with that in the right panel). These results suggest the presence of some variably disruptive influence acting within the GCL that perturbs cell positioning.

During early development, the cells in the GCL lie beneath the population of growing optic axons, invading retinal astrocytes and expanding blood vessels. Later on, as the eye continues to grow, the retina becomes progressively thinner, and these components of the innermost retina become intermingled with the cells in the GCL. We suggest that this “settling” of components amongst the cells of the GCL should produce displacements that shift cells about, degrading the patterning achieved during earlier stages (figure 7b, d) as cholinergic amacrine cells space themselves apart (Galli-Resta et al., 1997; Reese et al., 1999). The tendency for cells to be aligned along retinal vessels and optic fascicles in maturity is consistent with this, as is the physical displacement of single starburst amacrine cells relative to their dendritic stalks. Also supportive of this hypothesis is the increased packing efficiency and intercellular spacing observed in the *Bax*^{-/-} retina, where those innermost retinal components are maintained at a greater distance from the cholinergic mosaic in the GCL, due to the thicker GCL. Further detailed analysis of the spatial distribution of cholinergic amacrine cells across the GCL of the rabbit retina would be a particularly interesting test case for the present hypothesis, as it contains an avascular region associated with the visual streak, while the optic fiber layer contains large, myelinated fascicles of optic axons near the optic nerve head, where astrocytes and oligodendrocytes are preferentially distributed (Schnitzer and Karschin, 1986; Ehinger et al., 1994).

Acknowledgements

We thank Nicole Weber for assistance with data analysis.

Supported by the NIH (EY-11087)

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Figure 1.

a: Total retinal area (mean and standard error) for the two inbred laboratory strains, A and B6, and the four recombinant inbred (RI) strains, AXB4, AXB5, AXB24 and BXA26. b: Estimated total number of ChAT-immunoreactive amacrine cells in the GCL (filled bars) and INL (open bars) in these six strains of mice (same conventions hereafter). n = the number of retinas examined per strain, for both panels.

Figure 2. Examples of ChAT-immunoreactive mosaics in the GCL and the INL at the same retinal locus in an A retina and in a BXA26 retina. Note the higher density and the greater regularity in the INL relative to the GCL, being characteristic of all six strains. Calibration bar = 100 μ m.

Figure 3.

a: Average nearest neighbor regularity indexes (NNRI) for the GCL and INL for the six strains (left set of paired bars, conventions identical to figure 1), and average NNRI for random simulations matched for density and soma size (right set of shaded paired bars). Note that the NNRI for the INL is consistently larger than it is for the GCL. Furthermore, note that the NNRI for the INL is greater than that for random simulations, whereas the NNRI for the GCL is no better than a random distribution of cells. b: Average Voronoi domain regularity indexes (VDRI) for the GCL and INL mosaics and their matching random simulations. Again, the regularity of the INL mosaics is consistently higher than that for the GCL mosaics, but the mosaics in the GCL are now reliably more regular than the random distributions. c: Average packing factors (PF) for the GCL and INL mosaics and their matching random simulations. The INL mosaics are packed more efficiently than are the GCL mosaics, both being conspicuously different from random distributions of cells. d: The effective radius (ER) is larger in the INL, despite the INL having higher average densities of cells. Note that none of the average effective radii for the GCL is as low as would be predicted for random distributions of cells, in which the size of the effective radius is determined by soma size alone (10 μm). n = the number of fields sampled (an identical number of random simulations was therefore generated for the data in the right set of paired bars).

Figure 4.

a-d: Average NNRI (a), VDRI (b), PF (c) and ER (d) for the A strain GCL and INL mosaics shown in figure 3, alongside simulations of INL mosaics following random deletion of 15% of their cells (shaded bars). While both VDRI and PF are affected by this undersampling of the mosaic, neither the NNRI nor the ER is affected, indicating that the difference between the mosaics in the GCL and INL is not simply due to undersampling a mosaic with identical spatial properties. $n = 55$ sample fields in every histogram.

Figure 5.

a-c: Relationship between NNRI (a), VDRI (b) and PF (c) with density for the GCL (left) and INL (right). Both regularity and packing are largely preserved despite variation in density, for both layers. d: Relationship between ER and density for the GCL (left) and INL (right). Both layers show a significant inverse relationship with density, but there is conspicuously more variability in ER within the GCL at any given density. Data are drawn from the 237 individual fields that contributed the strain averages in figure 3.

Figure 6.

a, b: Cholinergic amacrine cells in the GCL (labeled for choline acetyltransferase, in green) nearly about the IPL stratum containing their dendritic processes at P3 (diagonal arrow in a), but become displaced from their starburst dendrites (diagonal arrow in b) by nuclear translocation through a dendritic stalk. The cholinergic amacrine cell on the left extends a dendritic stalk through the IPL which courses diagonally to the inner cholinergic plexus (horizontal arrow in b). Note the lesser segregation of cells from optic axons (labeled for neurofilaments, in red) in maturity relative to P3 (arrowheads in b and a, respectively), as well as the expansion of the thickness of the IPL with development. c, d: Cholinergic amacrine cells (green) frequently lie beneath blood vessels (labeled with PNA, in magenta) at P5 (arrows in

c) in this image from a retinal wholemount (central retina is to the top), but are more likely to be positioned aside vessels in maturity (diagonal arrow in d). Note the lack of any somata (labeled with NeuroTrace 640/660, in blue) between the unlabeled blood vessel (BV) and the IPL in d (horizontal arrow). e: Wholemount adult retina triple-labeled to show the population of cholinergic amacrine cells in the GCL (labeled for ChAT, in green), optic fascicles (labeled for neurofilaments, in red) and blood vessels (labeled with PNA, in blue). Note the prominent row of cholinergic cells aligned along a fascicle of optic axons (arrowheads). f: Drawing of a single filled cholinergic amacrine cell in the GCL displaced from the juncture of the dendritic stalk (arrow) and the center of its labeled dendritic field (red). Fascicles of optic axons (shown in light blue) course adjacent to, and occasionally over, the cholinergic amacrine cells (green), but retinal vessels (shown in grey) less commonly overlie these cells in maturity. g: Frequency distribution of the displacement distance of the centroid of single cholinergic amacrine cells from the position of their primary dendritic stalks at the level of the ON or OFF stratum where the radiating dendritic field emerges. h, i: Sections of retina from *Bax*^{+/+} (h) and *Bax*^{-/-} (i) mice showing the increased thickness of the INL and GCL in the *Bax*^{-/-} retina. The image in h has been sectioned transverse to the optic fascicles, while that in i has been sectioned parallel to their course. Calibration bar in f = 67 μm for a, b & d, = 160 μm for c, = 100 μm for e & f, and = 88 μm for h & i.

Figure 7.

a: Estimated total number (mean and standard error) of ChAT-immunoreactive amacrine cells in the GCL (filled bars) and INL (open bars) in the B6 strain at three different developmental ages (P3, P5 and P25). n = the number of retinas examined per age. b-d: Average NNRI (b), VDRI (c) and PF (d) for the GCL and INL as a function of developmental age in B6 retinas. Note that NN regularity (a) and packing efficiency (c) are higher at younger ages, and that the difference from adult values is greatest for the GCL mosaics when assessed for packing efficiency. n = the number of sample fields.

Figure 8.

a: Estimated total number (mean and standard error) of ChAT-immunoreactive amacrine cells in the GCL of *Bax*^{+/+} (filled bars) and *Bax*^{-/-} retinas (shaded bars). n = the number of retinas examined. b: Sample fields of cholinergic amacrine cells in the GCL in *Bax*^{+/+} and *Bax*^{-/-} retinas. c-f: Average NNRI (c), VDRI (d), PF (e) and ER (f) for in the GCL of *Bax*^{+/+} and *Bax*^{-/-} retinas. Both PF and ER were significantly increased. None of these measures differed in the INL. n = the number of fields sampled in c-f.

Primary antibodies used in the present study

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Antibody	Animal	Form	Dilution	Manufacturer	Antigen
Choline acetyltransferase	Goat polyclonal	Affinity purified immunoglobulin	1:50	Millipore, AB144P; Lot#0603023202 Temecula, CA	Human placental enzyme
GFAP-Cy3	Mouse monoclonal	Purified immunoglobulin	1:400	Sigma, C9205; Lot#085K4762 St. Louis, MO	Purified GFAP from pig spinal cord
Neurofilament 150kD	Rabbit polyclonal	Antisera	1:500	Millipore, AB 1981; Lot#0610042998	Purified bovine neurofilament polypeptide