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Dopaminergic Amacrine Cell Number, Plexus Density, and Dopamine Content in the Mouse Retina: Strain Differences and Effects of Bax Gene Disruption

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Abstract

Many types of retinal neuron modulate the distribution of their processes to ensure a uniform coverage of the retinal surface. Dendritic field area, for instance, is inversely related to the variation in cellular density for many cell types, observed either across retinal eccentricity or between different strains of mice that differ in cell number. Dopaminergic amacrine (DA) cells, by contrast, have dendritic arbors that bear no spatial relationship to the presence of their immediate homotypic neighbors, yet it remains to be determined whether their coverage upon the retina, as a population, is conserved across variation in their total number. The present study assessed the overall density of the dopaminergic plexus in the inner plexiform layer in the presence of large variation in the total number of DA cells, as well as their retinal dopamine content, to determine whether either of these features is conserved. We first compared these traits between two strains of mice (C57BL/6J and A/J) that exhibit a two-fold difference in DA cell number. We subsequently examined these same traits in littermate mice for which the pro-apoptotic *Bax* gene was either intact or knocked out, yielding a five-fold difference in DA cell number. In both comparisons, we found greater plexus density and DA content in the strain or condition with the greater number of DA cells. The population of DA cells, therefore, does not appear to self-regulate its process coverage to achieve a constant density as the DA mosaic is established during development, nor its functional dopamine content in maturity.

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Contributions

MS harvested retinal tissue and performed the immunolabeling, confocal imaging and cell counts; PWK conducted the plexus analysis; LH performed HPLC analysis; BER, PWK and PMI designed the study and wrote the manuscript.

Competing interests

All authors declare no competing interest.

Keywords

retinal mosaic; dendritic coverage; DA receptor; HPLC; cell death

INTRODUCTION

The neuromodulator dopamine plays critical roles during eye development and in regulating retinal function (Jackson et al., 2012; Zhou et al., 2017). The sole source of retinal dopamine is a population of amacrine cells situated in the inner nuclear layer (INL). These dopaminergic amacrine (DA) cells are the most sparsely distributed neurons within the retina (Versaux-Botteri et al., 1984; Whitney et al., 2009; Wulle and Schnitzer, 1989), comprising less than 0.01% of all cells (Keeley et al., 2014a). In the C57BL/6J (hereafter B6/J) mouse retina, their local distribution is highly irregular, if not random, because they minimize proximity to their closest neighbors but their density does not approach the maximum packing permitted by such spacing (Raven et al., 2003). Each DA cell in the mouse retina gives rise to a sparse if wide-field dendritic arbor in the inner plexiform layer (IPL), irregular in shape and bearing no spatial relationship to neighboring DA cells (Keeley and Reese, 2010a), as well as axonal processes that can span the width of the retina (Badea et al., 2009).

This lack of order in their mosaic, evidenced in both the spatial irregularity of their somata and in the absence of homotypic regulation by their immediate dendrites, should yield a non-uniform coverage of their processes across the retina, making them distinct from most other populations of retinal neurons (Reese and Keeley, 2015). Given the neuromodulatory nature of their role, however, regularity in somal patterning and uniformity of dendritic coverage may be features largely irrelevant to their function (Witkovsky, 2004), particularly in light of their extrasynaptic release and their volume transmission to affect DA receptors removed from the distribution of dopaminergic processes (Bjelke et al., 1996; Hirasawa et al., 2015; Nguyen-Legros et al., 1997; Puopolo et al., 2001; Veruki and Wässle, 1996). Curiously, the size of their cellular population is under precise if variable genetic control, as different strains of mice exhibit large inter-strain variation in their total number while exhibiting little intra-strain variation (Whitney et al., 2009). For instance, between the two inbred laboratory strains C57BL/6J and A/J, there is a ~2-fold difference in total number. The present study has asked whether the DA cells might still conserve their process coverage and retinal dopamine content in the presence of such large variation in the size of this neuronal population.

Because the C57BL/6J and A/J strains are known to differ in excess of five million sequence variants across their genomes, we also wanted to make such comparisons in the absence of conspicuous differences in genetic background. We consequently examined these same features between mice lacking the proapoptotic gene, *Bax*, and their littermate controls. In the absence of *Bax*, naturally occurring cell death is reduced, yielding a larger number of DA cells than are present in the control littermates (Keeley et al., 2012; Whitney et al., 2009). The mosaics and dendritic arbors of DA cells in *Bax*-KO retinas have previously been assessed, showing a spatial patterning approximating randomness, or even a tendency to clustering (Keeley et al., 2012), yet with dendritic field areas that do not scale in proportion

to the increase in cell number (Keeley and Reese, 2010a). As that former study considered only the dendritic fields of single labeled DA cells (Keeley et al., 2012), here we have compared the entire DA plexus in the IPL to determine whether it shows such compensatory regulation to maintain its density, and if DA content is similarly conserved.

MATERIALS and METHODS

Animals.

Eight mice from both the C57BL/6J (hereafter B6/J) and A/J inbred strains, and four *Bax*-knockout (*Bax*-KO) mice and four littermate control (*Bax*-WT) mice derived from mixed litters, were used for immunostaining. An additional eight mice of each inbred strain and six *Bax*-KO and *Bax*-WT mice were used for high-performance liquid chromatography (HPLC) analysis of dopamine. The *Bax*-knockout mice had been backcrossed for at least 10 generations with B6/J, though have been maintained by crossing heterozygous mice to produce *Bax+/+* (WT) and *Bax−/−*(KO) offspring. Mice for immunofluorescence were 42–49 days of age, while all mice for HPLC were 76 days of age. All mice were bred in the Animal Resource Center at UCSB, where they were maintained on a 12 hour light:dark cycle, with lights on at 7:00am, and maintained on the same shelf within the rack for each comparison. All experiments were conducted with approval by the UCSB Institutional Animal Care and Use Committee, and in accord with the NIH *Guide for the Use and Care of Laboratory Animals*.

Immunofluorescence.

Mice were given a lethal injection of sodium pentobarbital (Euthasol; 120mg/kg), and once deeply anesthetized, were intracardially perfused with 3 ml 0.9% saline followed by 50 ml of 4% paraformaldehyde in 0.1M sodium phosphate buffer (pH 7.2 at 20°C) containing 200 mM sucrose (Stradleigh et al., 2015). Retinas were dissected as wholemounts, taking care to ensure the entire retina was maintained intact, and subsequently immunostained using a sheep polyclonal antibody to tyrosine hydroxylase (Millipore; 1:10,000; Billerica, MA), and a donkey anti-sheep IgG conjugated to cyanine 3 (Jackson Immunoresearch; 1:200; West Grove, PA), all as previously described (Keeley et al., 2012).

Plexus density.

A single retina from each of four mice per group was sampled to analyze the dopaminergic plexus. For each retina, central and peripheral fields (~750 μm and ~1500 μm from the nerve head, respectively), being 120 μm^2 in area, were imaged in each of the four quadrants using an Olympus Fluoview 1000 laser scanning confocal microscope and a 60× objective. Image stacks were captured at 1 μm intervals through the depth of the entire IPL and the DA cell stratum of the INL. For each comparison, fields were assigned a random number in order to blind the experimenter to strain or genotype, after which fields were contrast-enhanced by eye using Adobe Photoshop (San Jose, CA) with the goal of matching the intensity of dendritic branches across all samples. These fields were then automatically converted into black and white images in Fiji (https://fiji.sc/) using the threshold function to generate the percent coverage of labeled pixels within the sampled field. The eight fields were combined

to generate an animal average, and the mean percent coverage and standard error of these animal averages, per group, is reported below.

Cell counting.

Retinas were subsequently examined on an Olympus BH2 microscope that was equipped for epifluorescence. With the aid of an attached video camera linked to a computer running Bioquant Nova software (Bioquant Image Analysis Corporation, Nashville, TN), a single retina from each mouse was outlined, and the position of every labeled cell was plotted across the entire retina, as described elsewhere (Keeley et al., 2017). Mean total DA cell numbers, as well as retinal areas, and their standard errors, per group, are reported below.

HPLC analysis.

Three hours after light onset, mice were euthanized with a lethal injection of sodium pentobarbital, and both eyes were immediately removed and their retinas dissected and frozen within a tube in dry ice. Retinal dopamine content was determined by high performance liquid chromatography (HPLC) with electrochemical detection. The samples were sonicated in a 100μL of ice cold 0.1 N $HClO_4$ containing 25 ng/ml 3,4-dihydroxybenzylamine (internal standard) and 0.01% sodium metabisulfite and centrifuged at 12,000 g for 15 min at 4°C. The supernatant fraction was transferred to HPLC autosample vials. The precipitate was re-dissolved in 0.1 N NaOH and assayed for protein (Lowry et al., 1951). The content of DA was determined as described (Pozdeyev et al., 2008). External standards of DA were analyzed in each experiment. Means and standard errors are reported below.

Statistics.

Means and standard errors are plotted in each histogram in the figures. Students' t-test was used for all comparisons, using a p value of < 0.05 for determining statistical significance. The p values for all comparisons are reported in the text, and those that are significant are indicated with a single asterisk in the figures.

RESULTS

B6/J versus A/J retinas.

DA cells in the mouse retina are all situated in the INL abutting the IPL, unlike some other mammalian retinas, where a sizeable number are displaced to the ganglion cell layer (GCL) (Eglen et al., 2003b; Oyster et al., 1985; Peichl, 1991). They have large somata relative to most other amacrine cells in the INL, and their overlapping processes blanket the retina, being most densely distributed in S1 of the IPL, but also extending into S3 and S5. As previously reported (Whitney et al., 2009), the number of DA cells in the retinas of B6/J and A/J mice is significantly different ($p = 2.3 \times 10^{-6}$), in the absence of any appreciable differences in retinal area ($p = 0.44$) (figure 1A-C). Their distributions appear comparably irregular (figure 1A), with the A/J strain confirmed to have roughly half the number of DA cells relative to B6/J (figure 1C).

Using confocal microscopy, we imaged the plexus of dopaminergic processes in the IPL of these two strains (figure 2A). By quantifying the proportion of each field occupied by DA processes, we generated a measure of "coverage" to describe the density of the plexus. Central and peripheral fields were generally comparable, and so the fields from the two eccentricities have been combined to generate an average across the eight sampled fields for each retina. Overall, we found that the plexus in the A/J strain was significantly sparser relative to that in the B6/J strain ($p = 0.03$), amounting to a 15% decrease (figure 2B). The DA cell mosaic in the mouse retina, therefore, does not appear to maintain any species-specific constancy of plexus coverage.

Using HPLC, we compared DA content between the two strains. Normalized DA content was significantly lower in the A/J strain compared to B/6J ($p = 0.0001$), showing a 25% decrease (figure 2C). Neither plexus density nor DA content, therefore, is conserved between the strains.

Bax-KO versus Bax-WT retinas.

Retinal area was slightly, though not significantly, larger in the Bax KO retina ($p = 0.13$), but the total number of DA cells in the *Bax*-KO retina was conspicuously increased relative to littermate control (*Bax*-WT) retinas (figure 3A). Counts of DA cells in the *Bax*-KO retinas showed a significant, ~5-fold, increase ($p = 6.9 \times 10^{-8}$) (figure 3B), as previously reported (Keeley et al., 2012). Those in the *Bax*-KO are even more irregularly distributed relative to control retinas, evidenced by their frequent side-by-side presence, with such close pairings occurring at a frequency at least as common as random simulations would predict, as previously demonstrated (Keeley et al., 2012).

The plexus of dopaminergic processes appeared denser, expected in light of the above strain comparison, in the *Bax*-KO retinas (figure 4A). The proportion of each field occupied by labeled pixels was significantly greater ($p = 0.002$), approximating a 30% increase (figure 4B). Here, as in the strain comparison, the more numerous DA cells in the *Bax*-KO retina establish a denser plexus, rather than maintaining a uniformity in process coverage. Analysis of DA content in these *Bax*-KO and *Bax*-WT control retinas showed a significant difference as well ($p = 7.9 \times 10^{-7}$), being 125% higher in the *Bax*-KO retina (figure 4C).

DISCUSSION

DA cells, via their processes, release dopamine that acts via volume transmission upon dopamine receptors that are widely distributed throughout the retina. Like all other retinal neurons, the population of DA cells exhibits comparably meager variability in their number within any strain, yet shows the greatest variability between different mouse strains across all retinal cell types studied to date (Keeley et al., 2014a). For instance, the ALS/LtJ strain has an average of 962 cells per retina while the AXB12 recombinant inbred strain has an average of 160 cells (Whitney et al., 2009). Given such conspicuous variability across strains, the present study asked whether this population regulates its process coverage to achieve a comparable blanketing of the retinal surface, potentially achieving a comparable dopamine content. Indeed, such a "density conservation principle" has recently been proposed for the dendritic arbors of 24 different types of retinal ganglion cell (Bae et al.,

2018). Retinal horizontal cells, that show a far more extensive degree of dendritic overlap than do retinal ganglion cells (having a coverage factor of ~6), exhibit a similar process occurring at the level of cone pedicles: the number of dendritic terminal endings extending into individual pedicles declines as a function of distance from the soma, suggesting a conservation of horizontal cell contacts at the individual pedicle (Reese et al., 2005). Both sets of studies imply a sensitivity to the presence of the processes of like-type cells. Our first study, having confirmed the two-fold difference in DA cell number between these two different mouse strains, B6/J and A/J, demonstrated that the DA plexus density in B6/J was significantly greater than in the A/J strain. While we cannot directly relate the difference in the absolute number of cells to the magnitude change in the plexus density measurements, because the processes of DA cells cross over one another, we can reasonably assume that, if these cells were capable of modulating their outgrowth in response to local homotypic density, we should have seen an unchanging plexus density. As we had previously reported that DA cells in the B6/J retina do not modulate their dendritic field sizes nor orientations in relation to immediate homotypic neighbors (Keeley and Reese, 2010a), we would conclude from the present study that the total dendritic plus axonal growth of a DA cell is not regulated by the density of other DA cells. A similar argument has been made for the processes of cholinergic amacrine cells, as this cell type does not modify its dendritic field size in relation to the local density of homotypic neighbors (Farajian et al., 2004; Keeley et al., 2007).

Given this difference in DA processes between the strains, one might still wonder if, through feedback inhibition, the dopamine content provided per cell might be down-regulated to yield comparable pan-retinal levels, via autoreceptors (Derouiche and Asan, 1999; Hadjiconstantinou et al., 1990; Nguyen-Legros et al., 1999). Instead, we found a significantly lower DA content in the A/J strain, the strain with fewer cells giving rise to an overall sparser plexus.

Of course, the factors that might participate in the self-regulation of the DA plexus and its dopamine content may be overshadowed by other genetic sources that modulate process outgrowth or dopamine metabolism (Vadasz et al., 2007). Those traits, like others already documented including cell number, mosaic spacing, or somal stratification (Keeley and Reese, 2014; Keeley et al., 2014b; Whitney et al., 2014; Whitney et al., 2011a; Whitney et al., 2009; Whitney et al., 2011b), may be susceptible to genetic variants, the effects of which might interfere with homotypic regulation. For this reason, we also examined *Bax*-KO and *Bax*-WT littermate retinas, as these mice should be on a comparable genetic background, and one that is congenic with the B6/J retinas used in our first study.

Indeed, our counts of DA cells in the B6/J and *Bax*-WT retinas were not significantly different, and nor were their retinal areas. By comparing *Bax*-KO with littermate *Bax*-WT retinas, the DA cells should now be genetically comparable, but for the lack of *Bax* and potentially any downstream genetic differences that might feasibly arise from this cell-intrinsic loss of *Bax*, or from the altered environment in which these DA cells now develop, given the lack of naturally occurring cell death in this and many other cellular populations. Regardless, this *Bax* comparison largely mimicked the primary features observed in the strain comparison, namely, a significant increase in dopaminergic plexus density in the *Bax*-

KO retina, and an even more conspicuous increase in retinal dopamine content. The increase in plexus density seen in the *Bax*-KO retina is similar to a report examining the effects of neurotrophin-3 (NT-3) overexpression (Yoshida et al., 2011). There, the authors reported significant increases in both cell number and in plexus density, consistent with the present results. However, as neurotrophins may have direct effects upon process outgrowth that are independent of how they modulate cell number (Calamusa et al., 2007; Cellerino and Kohler, 1997; Cellerino et al., 1998), the relationship between cell number and process density is obscured in that study. While neither that study (Yoshida et al., 2011) nor the present study compared the density of the far sparser distribution of dopaminergic processes that ascend to course in the outer plexiform layer, another study examining the *Bcl2*-transgenic mouse (in which this anti-apoptotic gene is overexpressed, yielding a 9-fold increase in DA cell number) reported a conspicuous (if not quantified) increase in the density of DA processes in the outer plexiform layer (Strettoi and Volpini, 2002). Together, that and the present study make clear that the density of neither set of processes is conserved.

As indicated above, the derived plexus density measure would not be expected to scale directly with either DA cell number or retinal DA content, though we would reasonably expect to measure a lack of a change if there was none. The incidence of process overlap is conspicuously greater in the Bax-KO retina, where those processes can also exhibit a degree of co-fasciculation (Keeley et al., 2012), yielding further underestimation of their frequency. We cannot rule out the possibility that process density isn't at least partially constrained by the increased number of cells, and the same may be said for the regulation of DA content, given that the magnitude increase of 125% in the *Bax*-KO retina, while large, is nothing as great as the increase in cell number (five-fold). What is clear from the present studies is that neither DA process coverage nor dopamine content is regulated at the population level to achieve a "conservation of density".

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Highlights:

- DA amacrine cell number varies considerably between different strains of mice
- The density of DA processes is significantly greater in retinas with more DA cells
- DA content is also significantly greater in retinas with more DA cells
- Neither DA process outgrowth nor DA content is regulated at the population level

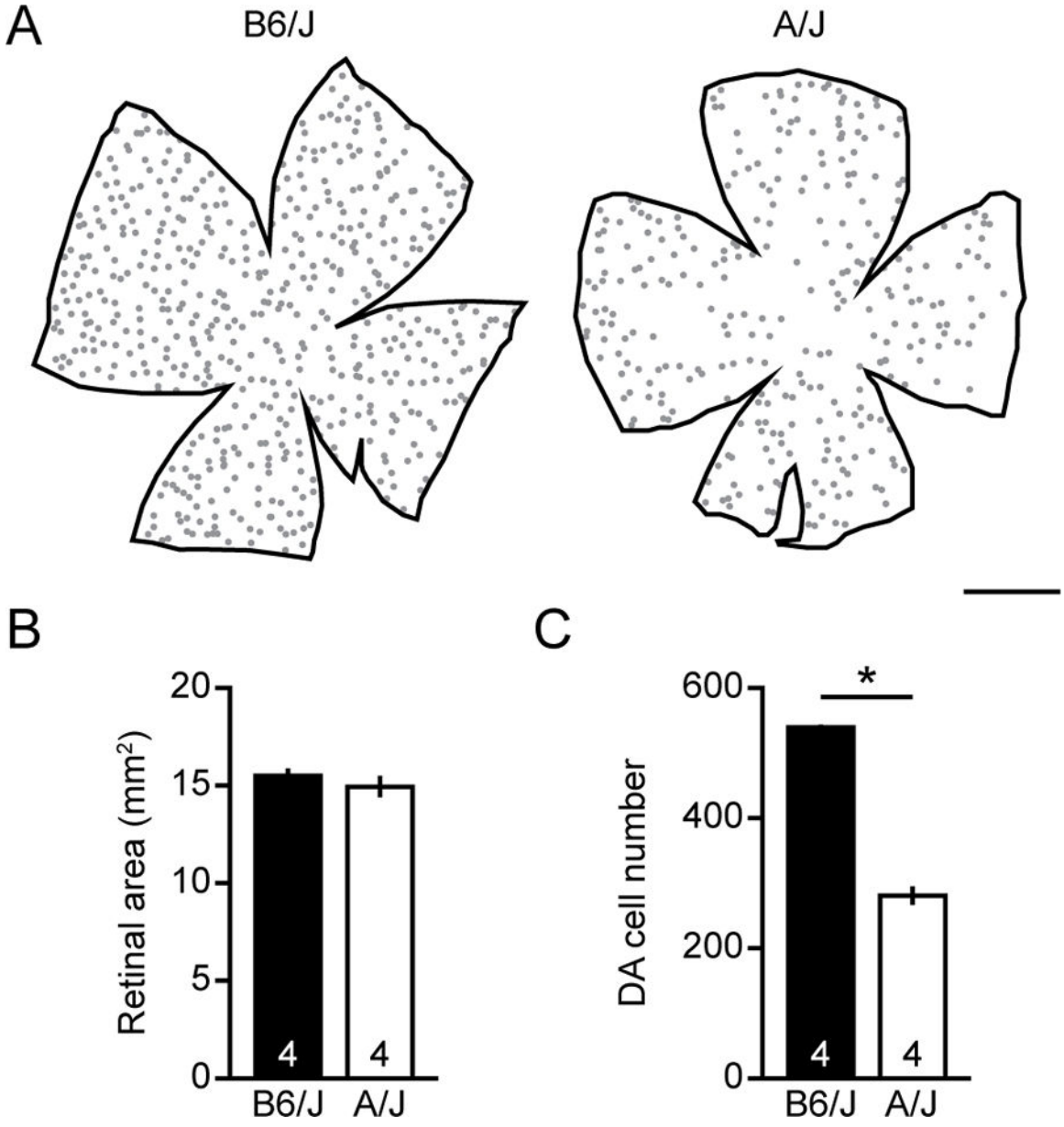

Figure 1.

A: Distribution of DA cells across the entire retina of a C57BL/6J and an A/J mouse. Scale bar = 1 mm. B and C: Total retinal areas and total DA cell numbers for four mice of each strain. Total cell number was significantly different between the two strains, with the A/J strain showing half the number observed in the B6/J strain, while retinal areal size was comparable.

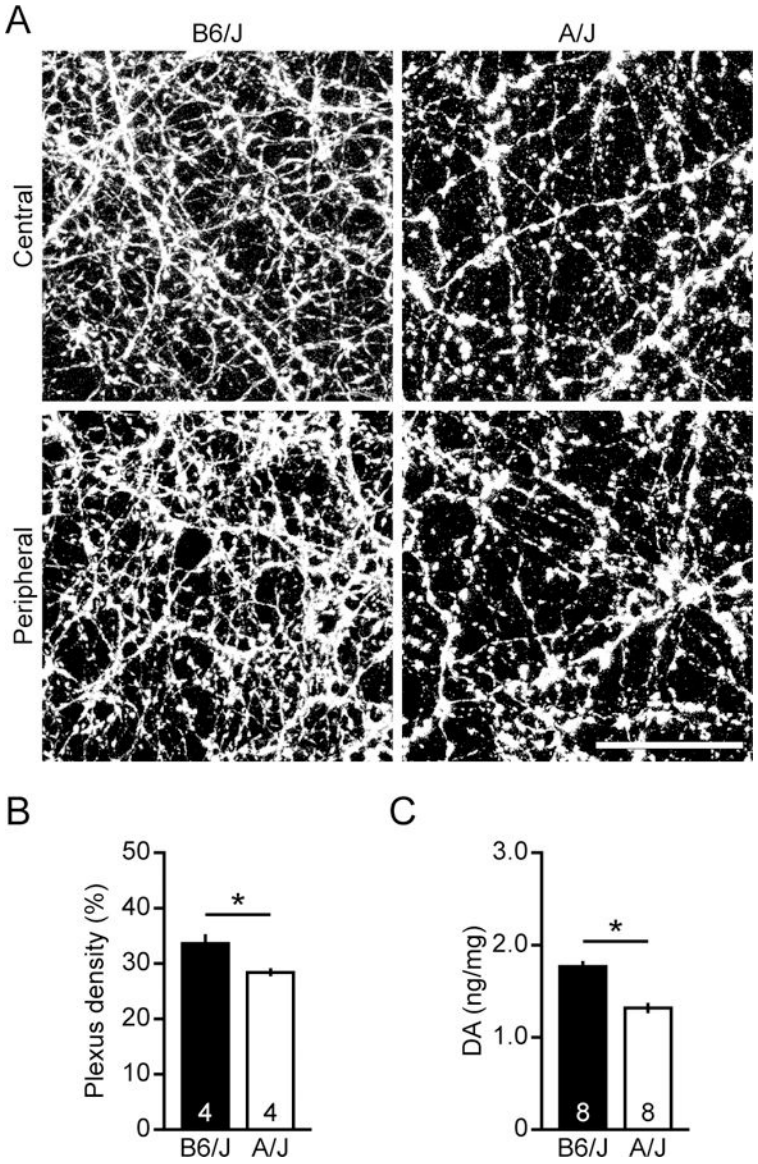

Figure 2.

A: Contrast-enhanced images used to estimate the degree of plexus coverage within a field (being the proportion of pixels in the image occupied by immuno-labeled processes), in both the B6/J and A/J strain retinas. Scale bar = 50 μm. B: Plexus coverage was significantly sparser in the A/J strain relative to the B6/J strain, showing a 15% decrease, derived from four mice in each strain. C: Total retinal DA content in the B6/J and A/J strains determined by HPLC analysis from eight mice in each strain. DA content was significantly lower in the A/J strain, being a 25% decrease.

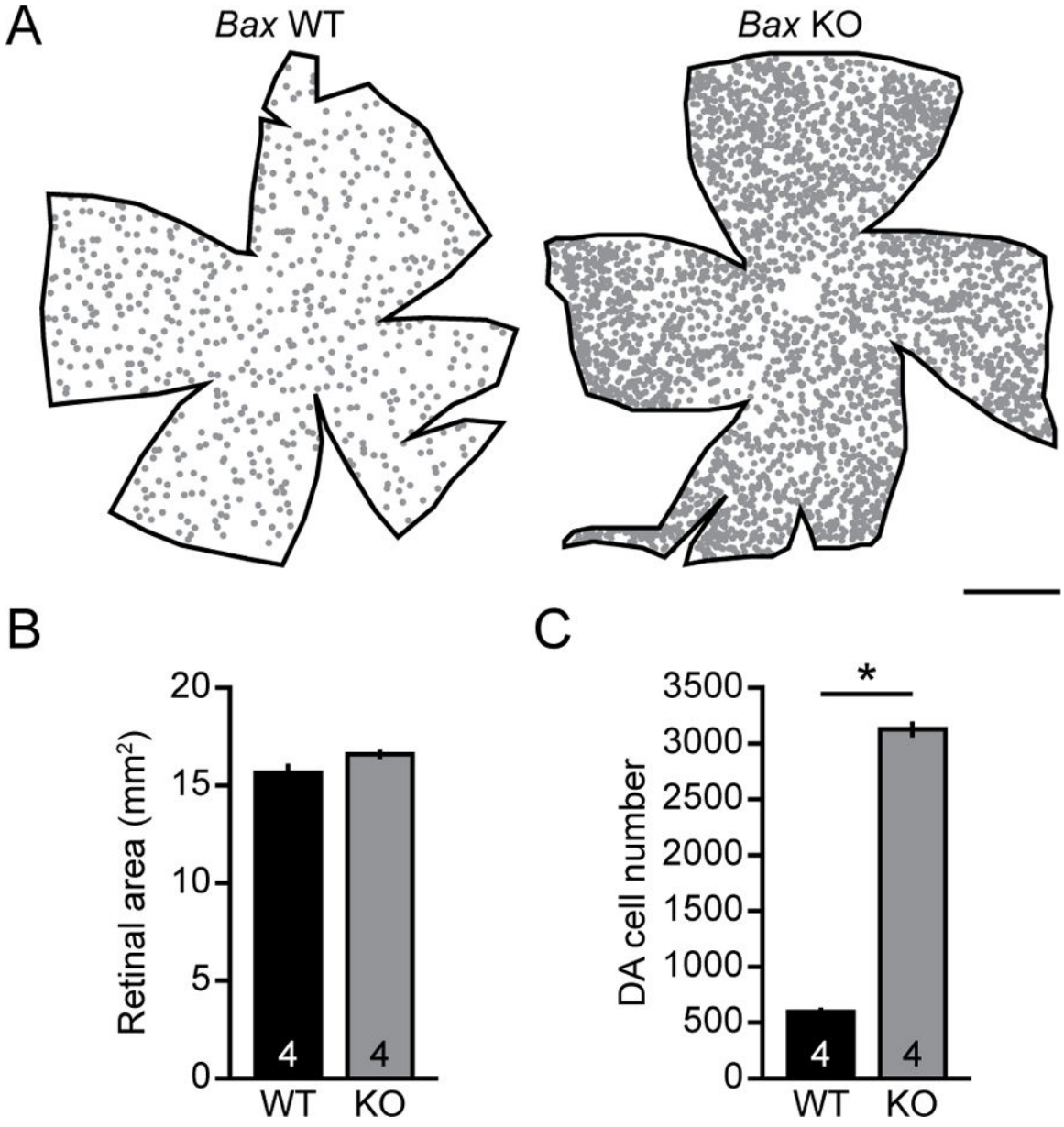

Figure 3.

A: Distribution of DA cells across the entire retina of a *Bax*-KO and *Bax*-WT littermate control mouse. Scale bar = 1 mm. B and C: Total retinal areas and total DA cell numbers for four mice of each condition. Total cell number was significantly different between the conditions, with the *Bax*-KO mice showing a ~5-fold increase in number, while retinal areal size was not significantly different.

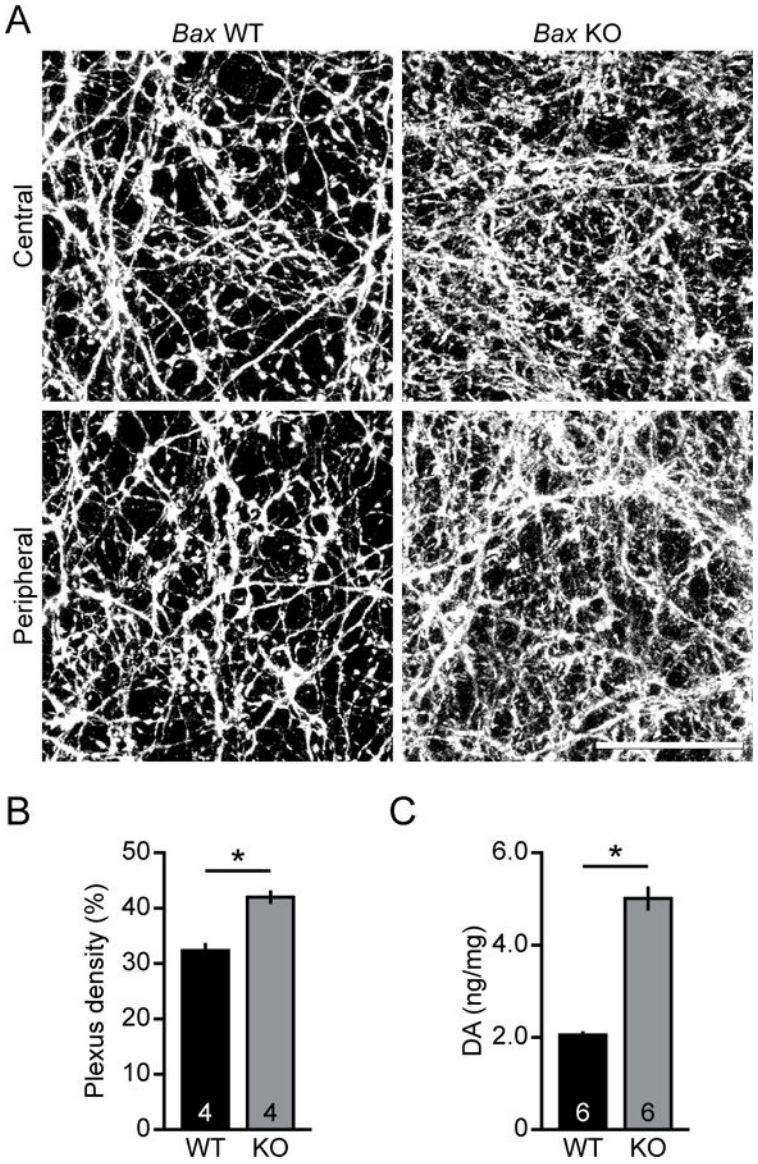

Figure 4.

A: Contrast-enhanced images used to estimate the degree of plexus coverage within a field (being the proportion of pixels in the image occupied by immuno-labeled processes), in both the *Bax*-KO and *Bax*-WT retinas. Scale bar = 50 μm. B: Plexus coverage was significantly greater in the knockout relative to littermate control mice, showing a 30% increase, derived from four mice in each condition. C: Total retinal DA content in the *Bax*-KO and *Bax*-WT retinas determined by HPLC analysis from six mice in each condition. DA content was significantly greater in the *Bax*-KO retina, being 125% higher than in *Bax*-WT.