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Disruption in dopaminergic innervation during photoreceptor degeneration

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Abstract

Dopaminergic amacrine cells (DACs) release dopamine in response to light-driven synaptic inputs, and are critical to retinal light adaptation. Retinal degeneration (RD) compromises the light responsiveness of the retina, and subsequently, dopamine metabolism is impaired. As RD progresses, retinal neurons exhibit aberrant activity, driven by AII amacrine cells, a primary target of the retinal dopaminergic network. Surprisingly, DACs are an exception to this physiological change; DACs exhibit rhythmic activity in healthy retina, but do not burst in RD. The underlying mechanism of this divergent behavior is not known. It is also unclear whether RD leads to structural changes in DACs impairing functional regulation of AII amacrine cells. Here, we examine the anatomical details of DACs in three mouse models of human RD, to determine how changes to the dopaminergic network may underlie physiological changes in RD. By using rd10, rd1 and rd1/C57 mice we were able to dissect the impacts of genetic background and the degenerative process on DAC structure in RD retina. We found that DACs density, soma size, and primary dendrite length are all significantly reduced. Using a novel adeno-associated virus-mediated technique to label AII amacrine cells in mouse retina, we observed diminished dopaminergic contacts to AII amacrine cells in RD mice. This is accompanied by changes to the components responsible for dopamine synthesis and release. Together, these data suggest that structural alterations of the retinal dopaminergic network underlie physiological changes during RD.

Keywords

dopamine; amacrine cell; retinal degeneration; gap junctions

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Role of authors

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: EI, CWY, BTS. Acquisition of data: EI. Analysis and interpretation of data: EI, CWY, BTS. Drafting of the manuscript: EI. Critical revision of the manuscript for important intellectual content: EI, CWY, BTS. Statistical analysis: EI, CWY. Obtained funding: BTS. Administrative, technical, and material support: EI, CWY, BTS. Study supervision: BTS.

Conflict of interest

All authors declare they have no conflict of interest.

Keywords

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Introduction

Visual perception requires the retina to operate across a wide range of light intensities. Dopamine, released by dopaminergic amacrine cells (DACs), is a chemical messenger that plays a pivotal role in this light adaptation (see Krizaj, 2000; Marshak, 2001; Witkovsky, 2004 for review). In healthy retina, dopamine release is stimulated by light and occurs in a circadian fashion (Gustincich et al., 1997; Ribelayga et al., 2008), playing an important role in modulating gap junction permeability, particularly in AII amacrine cells (Hampson et al., 1992). By controlling neuronal coupling via gap junctions, dopamine sets the gain and dynamic range of visual responses (reviewed by Bloomfield and Volgyi, 2009). Thus, changes to the light input of the retina may lead to alterations in dopamine metabolism and, in turn, to gap junction modulation.

Retinal degeneration (RD) begins with the atrophy of photoreceptors, impairing the light signal to the retina. Many neurons undergo anatomical and neurochemical remodeling (reviewed by Jones et al., 2012) and become hyperactive (Margolis et al., 2008; Stasheff, 2008). AII amacrine cells drive this activity, which propagates via gap junctions (Borowska et al., 2011; Choi et al., 2014; Menzler and Zeck, 2011). Surprisingly, DACs themselves are an exclusion to this shift towards hyperactivity, and in fact exhibit reduced bursting activity in RD (Atkinson et al., 2013), as well as reduced dopamine metabolism (Hankins and Ikeda, 1994; Nir and Iuvone, 1994). While physiological changes to DACs are well documented, the underlying mechanisms remain unclear. It is possible that these changes to DAC physiology, dopamine metabolism, and gap junction regulation may be linked to anatomical and neurochemical remodeling of DACs during RD, but this has not been previously explored. Indeed, structural changes to other inner retinal neurons are well-documented during RD. Retinal bipolar and horizontal cells undergo profound morphological alterations (Strettoi and Pignatelli, 2000) leading to functional abnormalities (Strettoi et al., 2002). Extensive changes in the morphology of both GABAergic and glycinergic amacrine cells are also evident at late stages of RD (Jones et al., 2003). Since DACs are the sole source of dopamine, a neuromodulator that plays a unique role in retinal function by modulating light adaptation, gain control of visual response, gap junction function, to name a few, as well as its potential role in maladaptive changes during disease, it is important to determine whether DAC structural integrity is preserved during RD.

This work aims at establishing the structural aspects of DACs and providing a necessary baseline for physiological assessment in the future. First, we examined DACs in rd1 and rd10 mouse retinas, which are models of fast and slow degeneration, respectively. Notably, the rd10 mouse model exhibits RD with later onset of photoreceptor cell loss with onset at P18, compared to rd1 with the RD onset at P8 (Gargini et al., 2007). Comparative analysis of these two mouse lines allowed us to distinguish whether alterations to DACs were caused

by developmental changes (rd1) or by remodeling of inner retinal neurons after the loss of the fully developed photoreceptor cells (rd10). Second, to determine the role of genetic background on retinal remodeling, structural changes to DACs in rd1 (C3H background) were compared to rd1/C57 (B6 background, provided by Dr. Bo Chang, the Jackson Laboratory, ME). We performed cell counts, measurements of soma size and dendritic density, as well as nuclear staining to reveal anatomical features of DACs. Additionally, contacts to AII amacrine cells were examined using a novel adeno-associated virus-mediated labeling method. Finally, immunohistochemical methods were used to visualize the components responsible for dopamine synthesis and release. We provide evidence for anatomical and neurochemical changes to DACs in RD and discuss how these may affect both regulation of AII amacrine cells and dopamine metabolism. These findings may provide new pathways and strategies for treating retinal degenerations.

Materials and Methods

Animals

In all experimental procedures, animals were treated according to the regulations in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, in compliance with protocols approved by the Institutional Animal Care and Use Committee of Weill Cornell Medical College, and in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Homozygous mice of either sex were obtained from the Jackson Laboratory (Bar Harbor, ME). Four mouse lines were used: rd10 (RRID:Jax:004297), rd1 (RRID:Jax:000659), rd1/C57 (B6.C3-Pde6brd1 Hps4le/J, RRID:Jax:000002) and wild type mice (C57BL/6J, RRID:Jax:000664).

Viral injections

AII amacrine cells were visualized by injection of a recombinant adeno-associated virus serotype 2 (rAAV2) carrying a construct of green fluorescent protein (GFP) under control of a cytomegalovirus (CMV) promoter as previously described (Ivanova and Pan, 2009). The rAAV2 carries a Y444F capsid mutation for highly efficient vector transduction (Petr-Silva et al., 2009). Briefly, mice aged postnatal day 30–60 (P30–60) were anesthetized by intraperitoneal injection of a mixture of 150 mg/kg ketamine and 15 mg/kg xylazine. Under a dissecting microscope, a small perforation was made in the temporal sclera region with a sharp needle. A total of 1.5 µl viral vector suspension in saline was injected into the intravitreal space through the perforation with a glass pipette (1B150F-4; WPI, Sarasota, FL) pulled with a P-97 Flaming/Brown puller (Sutter Instruments, Novato, CA). Viral vectors were packaged and affinity purified by Virovek (Virovek, Hayward, CA).

Immunohistochemistry

Mice aged P180 were deeply anesthetized with CO₂ and killed by cervical dislocation. Eyes were enucleated, retinas were fixed in the eyecups with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB, pH = 7.3) for 25 minutes. Next, the retinal temporal quadrant was identified based on choroid marks (Wei et al., 2010) and was marked by a half-radius incision. For immunostaining, retinal wholemounts were dissected from the eyecup and were blocked for 1 h in a solution containing 5% Chemiblocker (membrane-blocking agent,

Chemicon), 0.5% Triton X-100, and 0.05% sodium azide (Sigma). Primary antibodies were diluted in the same solution and applied for 72 h, followed by incubation for 48 h in the appropriate secondary antibody, conjugated to Alexa 568 (1:1000; red fluorescence, Molecular Probes), Alexa 488 (1:1000; green fluorescence, Molecular Probes), Cy3 (1:1000; red fluorescence, Jackson ImmunoResearch). In multi-labeling experiments, tissue was incubated in a mixture of primary antibodies, followed by a mixture of secondary antibodies. All steps were carried out at room temperature. After staining, the retina was flat-mounted on a slide ganglion cell layer up, and cover slipped using Vectashield mounting medium (H-1000, Vector Laboratories). The coverslip was sealed in place with nail polish. Small pieces of a broken cover slip glass (Number 1 size) were placed in between the slide and the coverslip glasses to avoid extensive squeezing and damage to the retina. The GFP fluorescence was sufficient to visualize the GFP-expressing neurons, without antibody enhancement. All primary antibodies used in this study are listed in Table 1.

Anti-tyrosine hydroxylase (TH) antibody was used to label dopaminergic amacrine cells (ImmunoStar Cat# 22941 RRID:AB_572268). The antibody recognizes a characteristic band of 62kDa on PC12 lysates. Our retinal labeling was similar to the staining pattern described by other investigators (Dumitrescu et al., 2009).

The antibody against VMAT2 (PhosphoSolutions, RRID: AB_2315595) was raised in sheep against a peptide from the C-terminal tail (C-TQNNIQSYPIGEDEEESD-OH) as previously described (Haycock et al., 2003), and specificity has been confirmed by a substantial (>85%) decrease of immunoreactivity in striatum from Parkinson's disease subjects. According to the manufacturer, the antibody recognizes a specific band of the 57kDa VMAT2 protein by Western blot on rat caudate lysate. The staining pattern was identical to retinal labeling achieved by other investigators (Witkovsky, 2004; Witkovsky et al., 2005). For nuclear staining, we used TO-PRO-3 iodide (642/661; Invitrogen; T3605; 1:1000), a highly sensitive probe for double-stranded nucleic acids. TO-PRO-3 was co-applied with secondary antibodies.

Data acquisition and analysis

Confocal Z-stacks were taken from retinal wholemounts using a Nikon Eclipse Ti-U confocal microscope at different magnifications and retinal regions (Fig. 1). The final magnifications were chosen to match the size of the measurable structures e.g. small axon varicosities were studied with a $\times 60$ oil objective. The retinas from all mouse lines were imaged under identical acquisition conditions, including: laser intensity, photomultiplier amplification, and Z-stack step size. Five retinas from different mice of each line were examined in any given experiment (unless noted otherwise). Images were processed and were analyzed using ImageJ software (ImageJ, RRID:nif-0000-30467). The resulting data are presented as mean $\pm$ standard error. Statistical analyses were performed using a one-way or two-way Analysis of Variance (ANOVA) in SigmaPlot (SigmaStat, RRID:nlx_157306) or SPSS (SPSS, RRID:rid_000042, IBM).

Distribution of DACs was automatically analyzed at each quadrant after the cells' somas were recognized and counted with the particle recognition algorithm. Primary dendrites were identified based on their morphological properties and were manually traced. Their

densities were calculated as the total length divided by the area where dendrites were traced. The soma size was evaluated in the same areas. Each cell body was manually traced along its perimeter and the areas within were calculated.

Fine axonal systems of dopaminergic cells were studied in 12 areas (three for each quadrant, Fig. 1, black boxes). Within each of the 12 areas two $35\text{ }\mu\text{m} \times 35\text{ }\mu\text{m}$ single confocal sections were taken for analysis. Small areas allowed us to have the axonal system in focus within the entire area and to apply automatic recognition and evaluation of the TH- and VMAT2-positive structures. For the automatic recognition the global thresholds were first empirically established and then applied to all images. After that the particle recognition algorithm was applied and the number, size and labeling intensities of all recognized particles were estimated.

Contacts between TH-positive processes and AII amacrine cells were visualized with BioView3D and quantified with ImageJ. Confocal image stacks were processed one slice at a time. The somas of GFP-expressing AII amacrine cells were targeted and surrounding TH + processes were identified. Contacts were defined as processes that were within $1\text{ }\mu\text{m}$ of the soma. Points of contact were demarcated with the line tool and lengths were calculated. Surface area was interpolated by multiplying total length for a given slice by the z-depth of the slice.

Rotated projections of image Z-stacks $50\text{--}70\text{ }\mu\text{m}$ thick were generated by ImageJ. Images of AII amacrine cells with the contacts of dopaminergic cells were processed in ImageJ and bioView3D (Center for Bio-Image Informatics, Santa Barbara, CA).

Results

Retinal degeneration results in abnormalities in dopamine metabolism (Doyle et al., 2002b; Hankins and Ikeda, 1994; Nir and Iuvone, 1994; Park et al., 2013), and altered physiology of DACs (Atkinson et al., 2013). The experiments described here examine anatomical differences between DACs in wildtype (wt), rd10, rd1, and rd1/c57 mouse retina. DACs were identified by labeling for tyrosine hydroxylase (TH), the enzyme responsible for the conversion of tyrosine to L-DOPA, the precursor of dopamine. The following data provide evidence that, in RD, (1) DACs are morphologically compromised; (2) the components responsible for dopaminergic output are altered, including (3) a reduction in DAC processes associated with AII amacrine cells.

Degradation of DACs and their primary dendrites in RD

First, we performed counts of DACs in wt, rd10, rd1, and rd1/c57 retinas to determine differences in total population (Fig. 2A–D). The number of DACs in rd1 retina (436 ± 13 ; $n = 3$, $p = 0.003$) was significantly lower than in rd1/c57 (642 ± 21 , $n = 3$), wildtype (613 ± 38 , $n = 5$) and rd10 (650 ± 18 , $n = 5$) retinas (Fig. 2E). There was no significant difference between the pairs of wildtype and rd10 ($p = 0.35$) and wt and rd1/c57 ($p = 0.53$) DAC counts. We then determined whether the distribution of DACs varied with retinal region. In wt mice, these cells form a mosaic organization, with even distribution across retinal quadrants (Raven et al., 2003). There was a significant difference in cell counts between

strains, with an additional effect of retinal region (two-way ANOVA, main effect of strain, $p = 0.002$, with a significant interaction with region, $p = 0.044$). rd10 and rd1/c57 did not differ from wt at any given region ($p > 0.57$ for each). The cell count in rd1, however, was significantly lower than wt in dorsal ($p = 0.011$) and temporal regions ($p = 0.006$), but no different in ventral or nasal regions ($p = 1.0$).

Next, we examined more detailed anatomical changes focusing on soma sizes and the primary dendrites (Fig. 3). Soma sizes of rd10 ($124.19 \pm 0.91 \mu\text{m}^2$), rd1 ($132.24 \pm 1.97 \mu\text{m}^2$), and rd1/C57 ($136.97 \pm 1.49 \mu\text{m}^2$) cells were significantly smaller than wt ($178.08 \pm 1.67 \mu\text{m}^2$; $p < 0.0001$ for all, Fig. 3E–H, Fig. 3M–P and Fig. 3Q). There were variations in size across retinal poles for all three genotypes ($p < 0.0001$), and this variation was consistent between them (2-way ANOVA, no significant interaction of genotype $\times$ pole, $p = 0.23$). At all poles, wt soma sizes (in μm^2 – dorsal: 193.30 ± 4.04 , $n = 117$; nasal: 184.08 ± 2.95 , $n = 134$; temporal: 164.28 ± 2.98 , $n = 139$; ventral: 173.02 ± 3.00 , $n = 132$) were greater than rd10 (dorsal: 124.91 ± 1.84 , $n = 195$; nasal: 130.06 ± 2.05 , $n = 182$; temporal: 120.18 ± 1.60 , $n = 189$; ventral: 121.91 ± 1.73 , $n = 197$), rd1 (dorsal: 134.08 ± 3.17 , $n = 90$; nasal: 147.28 ± 3.93 , $n = 85$; temporal: 115.96 ± 2.86 , $n = 65$; ventral: 125.49 ± 4.94 , $n = 57$), and rd1/C57 (dorsal: 152.56 ± 2.76 , $n = 74$; nasal: 129.96 ± 2.98 , $n = 96$; temporal: 140.82 ± 3.07 , $n = 78$; ventral: 130.34 ± 2.56 , $n = 118$) ($p < 0.0001$ in all comparisons). rd1/C57 soma sizes were greater than rd10 and rd1 at dorsal ($p < 0.0001$, $p = 0.0013$) and temporal ($p < 0.0001$ for both) poles, but no different at the ventral and nasal poles ($p = 0.086$, $p = 0.89$). rd1 soma size was greater than rd1/C57 and rd10 at the nasal pole ($p = 0.0014$, $p = 0.00021$).

We also examined primary dendrites (Fig. 3A–D, Fig. 3I–L and Fig. 3R), which are large, thick, and irregularly shaped processes radiating from the soma that allow DACs to receive synaptic input (Dacey, 1990). In wt retinas the primary dendrites were thick, prominently labeled, and visible for a long distance from the soma. Usually, three to five primary dendrites could be detected from each DAC soma in wt. In contrast, in all RD retinas they were thinner and weaker labeled and could not be easily distinguished from the surrounding fine axonal net. As a result, in RD fewer dendrites were detected for each DAC and those dendrites could be traced for shorter than in wt distances from somas. Among RD retinas the morphology of primary dendrites was best preserved in rd10, followed by rd1/c57 and rd1 retinas, which is reflected by the statistical comparison. Primary dendrite densities (mm per 0.4 mm^2) in rd10 (3.8 ± 0.21 , $n = 5$ animals, 20 areas), rd1 (2.05 ± 0.45 , $n = 3$ animals, 12 areas), and rd1/C57 (2.73 ± 0.23 , $n = 3$ animals, 12 areas) retinas were again significantly less than wt (5.79 ± 0.30 , $n = 5$ animals, 20 areas $p < 0.0001$ in all cases). The density in rd1 was also significantly less than rd10 ($p = 0.0008$), but rd1/C57 was not significantly different from either rd10 ($p = 0.081$) or rd1 ($p = 0.67$).

Interestingly, in wt and especially in RD mice the morphology of the primary dendrites varied among the quadrants of the same retina (compare Fig. 3A–D and 3I–L). In all lines at the dorsal retinal pole the dendrites were strongly labeled, thicker and longer. When comparing across poles, we found that wt densities (dorsal: 6.91 ± 0.40 ; nasal: 6.42 ± 0.59 ; temporal: 5.22 ± 0.31 ; ventral: 4.59 ± 0.46) were significantly greater than rd10 in all but the ventral pole (dorsal: 4.63 ± 0.45 , $p = 0.012$; nasal: 4.04 ± 0.31 , $p = 0.0091$; temporal: $2.83 \pm$

0.30, $p = 0.0003$; ventral: 3.83 ± 0.22 , $p = 0.53$), and greater in all poles than in rd1 (dorsal: 3.65 ± 0.05 , $p = 0.0023$; nasal: 3.34 ± 0.45 , $p = 0.0038$; temporal: 0.72 ± 0.13 , $p < 0.0001$; ventral: 0.50 ± 0.34 , $p < 0.0001$), and rd1/C57 (dorsal: 3.58 ± 0.68 , $p = 0.0019$; nasal: 2.65 ± 0.12 , $p = 0.00071$; temporal: 2.51 ± 0.33 , $p = 0.00035$; ventral: 2.19 ± 0.29 , $p = 0.0039$). rd1 densities were less than in rd10 in the temporal and ventral poles ($p = 0.0031$, $p = 0.00024$), but not the dorsal and nasal poles ($p = 0.67$, $p = 0.90$). rd1/C57 densities were less than rd1 in the temporal pole ($p = 0.023$) but were otherwise not significantly different from either rd1 (dorsal: $p = 1.0$; nasal: $p = 0.94$; ventral: $p = 0.085$) or rd10 (dorsal: $p = 0.59$; nasal: $p = 0.31$; temporal: $p = 0.98$; ventral: $p = 0.054$). There were main effects of genotype ($p < 0.0001$) and pole ($p < 0.0001$), with a significant interaction between the two (2-way ANOVA, $p = 0.037$).

To better visualize changes in the soma of DACs, we double-labeled the cells using a nuclear marker (TO-PRO-3, Fig. 4). This marker specifically stains chromatin, and may thus be used to visualize chromatin condensation, an indicator of cells undergoing apoptosis (Chang et al., 1997; Kerr et al., 1972; Quigley et al., 1995; Wyllie et al., 1981). Across mouse strains, the intensity of the TO-PRO-3 signal was weaker in DAC nuclei relative to those of surrounding amacrine cells. Staining in wildtype nuclei was diffuse, with 4–5 spots of higher intensity; rd10 nuclei had a similar diffuse staining, but only had 1–2 spots of higher intensity, which were larger than those observed in wt nuclei. rd1 and rd1/c57 nuclei lacked diffuse staining, and only had 1–2 large spots of higher intensity. The cytoplasm of DACs, revealed by TH immunolabeling, occupied a smaller volume and was stained weaker in RD in comparison to wt.

Changes in the axonal system and sites of vesicular release in DACs

We next evaluated sites of dopaminergic output in DACs. The axonal system of DACs is comprised of an elaborate net of processes that extend from soma and bear distinct varicosities at sites of synaptic output (Dacey, 1990; Kolb et al., 1990). In confocal images from retinal wholemounts we evaluated the axonal system of DAC in sublamina 1 of the IPL where it directly contacts AII amacrine cells (Fig. 5A–D). In wt, the axonal system was represented by a dense net of slender processes and varicosities, both were well immunolabeled against TH. In rd10 and rd1/c57 retinas, the staining appeared to be more fragmented. The fragmentation was even more obvious in rd1 retina, where TH labeling was concentrated in varicosities and was very low in the connecting slender processes. To quantify the fragmentation we applied the particle recognition and fluorescence intensity threshold procedures to DAC structures. Larger particles were detected in wt where both varicosities and the slender processes connecting them were intensely (above threshold) labeled for TH. When quantified, the size of the detected particles was significantly larger in wt animals ($5.5 \pm 0.44 \mu\text{m}^2$, $n = 60$) compared to rd10 ($1.6 \pm 0.083 \mu\text{m}^2$, $n = 52$, $p < 0.001$), rd1 ($1.0 \pm 0.059 \mu\text{m}^2$, $n = 36$, $p < 0.001$), and rd1/C57 ($2.8 \pm 0.15 \mu\text{m}^2$, $n = 36$, $p < 0.001$) mice (Fig. 5M). rd1/c57 particles were larger than in both rd10 ($p = 0.028$) and rd1 ($p = 0.0014$). rd10 did not differ from rd1 ($p = 0.78$). In rd10, rd1/c57 and particularly in rd1, TH immunoreactivity was more prominent in varicosities and at a much lower level (often below threshold) in the slender processes.

Next, we visualized sites of vesicular dopamine release using a presynaptic marker for vesicular monoamine transporter 2 (VMAT2; Witkovsky, 2004), allowing vesicles packed with dopamine to be visualized as puncta on DAC axonal processes (Fig. 5E–H). In the retina, the maturation of VMAT2 particles occurs late in development (Witkovsky et al., 2005), therefore it could be affected by RD. In rd1, VMAT2 immunofluorescence was confined to DAC varicosities (Fig. 5I–L). The size of VMAT2 puncta (Fig. 5N) was significantly larger in rd1 ($0.93 \pm 0.062 \mu\text{m}^2$, $n = 36$) compared to wt ($0.46 \pm 0.015 \mu\text{m}^2$, $n = 60$, $p < 0.0001$), rd10 ($0.54 \pm 0.014 \mu\text{m}^2$, $n = 52$, $p < 0.0001$), and rd1/C57 ($0.47 \pm 0.0088 \mu\text{m}^2$, $n = 36$, $p < 0.0001$). The large size and fewer particles in rd1 probably reflect their immature state, as has been shown for rat retina (Witkovsky et al., 2005). The VMAT2-positive particles were similar in wt, rd10, and rd1/C57 ($p > 0.23$ for each comparison).

Reduction in DAC contacts to AII amacrine cells

AII amacrine cells are a major target of dopaminergic amacrine cells (Contini and Raviola, 2003; Pourcho, 1982; Voigt and Wassle, 1987), and are primary contributors to the aberrant physiological activity that occurs during RD (Borowska et al., 2011; Choi et al., 2014; Margolis et al., 2014; Yee et al., 2012). Processes of dopaminergic amacrine cells have been shown to form ring-like structures around the soma of AII amacrine cells in several animal models (Vaney, 1985; Voigt and Wassle, 1987), including mouse (Rice and Curran, 2000). Here, we examine DAC cell processes in relation to AII amacrine cells in RD retina.

To visualize AII amacrine cells, we used a genetic approach that utilizes rAAV-mediated GFP expression (see Methods) and provides superior visualization of AII amacrine cell processes than commercially available antibodies to Disabled-1 (Ivanova and Pan, 2009). Within the INL, GFP expression was predominantly localized in cells with characteristic morphological properties of AII amacrine cells: soma with an emerging single primary dendrite that branches to form lobular appendages in the OFF sublamina and arboreal dendrites in the ON sublamina (Fig. 6A, inset). Expression was also found in the ganglion cell layer (GCL, data not shown). DAC processes were visualized in the same preparations (Fig. 6D–F). As observed in previous studies, dopaminergic ring structures were mostly concentrated in strata 1 of the IPL, around the soma of AII ACs (Fig. 6G–I). The number of proximal DAC processes were visibly reduced in rd10 and rd1 compared to wt. To quantify these differences, individual AII cells and proximal DAC processes were reconstructed in 3D from Z-stacks (Fig. 7A–F). Points of contact between DAC processes and AII cells occurred primarily around soma and lobular appendages. DAC contacts on arboreal dendrites were rare and are not shown. The area of contacts was significantly different between strains (Fig. 7G, ANOVA, $p < 0.001$). The area of contacts to wildtype cells ($128.54 \pm 8.24 \mu\text{m}^2$, $n = 6$) was significantly greater than both rd10 ($48.68 \pm 7.02 \mu\text{m}^2$, $n = 6$, $p = 0.002$) and rd1 ($34.35 \pm 1.98 \mu\text{m}^2$, $n = 6$, $p < 0.001$), but RD strains did not differ from each other ($p = 0.89$). Although we did not evaluate the morphology of AII ACs in detail, it seems that the lobular appendages were longer in RD mouse strains.

Discussion

The present study documents alterations of dopaminergic cells in an advanced stage of RD. We found that DACs in RD retina were reduced in number, soma size, and dendrite density. Expression levels of TH and VMAT2 were reduced and had altered spatial distribution, providing evidence for compromised dopamine metabolism and release. By using rd10, rd1, and rd1/C57 mice, we were able to dissect the impacts of genetic background and the degenerative process on DAC structure in RD retina. The functional implications of these anatomical and neurochemical changes in RD are discussed below.

DAC structure and function in degenerated retina

Levels of retinal dopamine are decreased in dystrophic retinas, but the underlying mechanisms remain unclear (Hankins and Ikeda, 1994; Nir and Iuvone, 1994). Our results suggest that altered dopaminergic physiology and metabolic function are related to gross anatomical changes to DACs. DACs were analyzed in rd10, rd1 and rd1/C57 retinas, models of fast and slow RD progression, respectively (Gargini et al., 2007). Accordingly, changes to DACs were consistently greater in rd1 compared to rd10, with rd1/C57 in the middle. It is possible that the early onset of degeneration in rd1 may interfere with the development and maturation of DACs, as this has been found in retinal ganglion cells (Damiani et al., 2012). The fragmented TH-labeling, indistinguishable primary dendrites, and large sparse VMAT2-positive structures found in our rd1 retina, are characteristic for the early stages (P13-P19 in rat) of DAC development (Witkovsky et al., 2005). This is in contrast to our findings in rd10 and rd1/C57, in which the onset of the RD starts at later stages of development (discussed below). The chromatin condensation observed in all RD strains suggests that the anatomical and neurochemical changes observed in DACs are also due to stress (Chang et al., 1997; Yu et al., 2002).

Light-driven input may be essential to the survival of DACs. The majority of DAC contacts are output contacts of the axonal system (Dacey, 1990; Kolb et al., 1990; Pourcho, 1982). Excitatory input contacts, meanwhile, are relatively few, and originate from ON cone bipolar cells and melanopsin-expressing ganglion cells (Newkirk et al., 2013; Zhang et al., 2008; Zhang et al., 2007). Our results showed a preferential survival of DACs at the dorsal quadrant of the retina (Fig. 2), as well as preservation of their structural components compared to the ventral quadrant. Interestingly, in rd1, light responses of surviving DACs are mediated primarily by melanopsin phototransduction, rather than cones (Atkinson et al., 2013). In both wt and rd retinas, M1 and M2 melanopsin cells are more concentrated dorsally (Hughes et al., 2013); the survival of DACs in the dorsal quadrant may therefore be related to persisting light-driven input from these melanopsin cells. This relationship between residual light input and dopaminergic cell survival would be of interest for further study.

The morphological changes we observe in DACs may be related to their decline in function. Tyrosine hydroxylase (TH) is the principal enzyme in dopamine synthesis, and its expression is generally correlated to dopamine levels (Iuvone et al., 1978; Sved et al., 1984). The decreased expression of TH we observe is in agreement with the altered dopamine metabolism previously found in RD retina (Frucht and Melamed, 1984; Nir and Iuvone,

1994; Park et al., 2013). Additionally, TH was concentrated in the varicosities of DACs, particularly in rd1, in contrast to wt, in which TH is more evenly distributed throughout the processes of DACs. VMAT2, which indicates sites of dopamine release, is concentrated at varicosities across strains. Thus, as RD progresses, dopamine synthesis may become restricted to synaptic sites of release. This is in contrast to wt, in which neurotransmitter release from DACs also occurs extrasynaptically, distributed throughout the cell (Hirasawa et al., 2009; Puopolo et al., 2001), which could lead to accumulation of neurotransmitter within the cell. This is evidenced by increased VMAT2 expression in rd1 DACs (Fig. 5) and indicative of a concomitant increase in the quantal size of dopamine vesicles (Pothos et al., 2000), though evidence suggests that this does not translate to increased frequency of release (Hankins and Ikeda, 1994; Nir and Iuvone, 1994). Indeed, DACs are polyaxonal (Dacey, 1990) and dopamine release is activity-dependent, directly related to action potential generation (Puopolo et al., 2001). The reduced bursting activity of RD DACs (Atkinson et al., 2013) therefore suggests that these vesicles are released less frequently than in wt. It is possible that the lowered release frequency, combined with the lack of extrasynaptic release, may lead to larger vesicles of dopamine due to accumulation.

Genetic background influences RD remodeling

We found that changes in DACs were more severe in rd1 compared to rd1/C57 retina. Since both strains carry the same mutation to Pde6b, this difference is likely due to the difference in genetic background (C3H vs C57). The rate of degeneration may thus be due not only to mutation, but can be accelerated or impeded by genetic background. Indeed, it has previously been found that aberrant activity develops more slowly in rd1 on a C57 background compared to rd1 on a C3H background, at a rate more similar to rd10 (Stasheff, 2008; Stasheff et al., 2011). One possibility for this effect of genetic background (C57 vs C3H) on the RD is different levels of melatonin: C57 mice do not have detectable amounts of melatonin, while C3H mice do (Ebihara et al., 1986; Goto et al., 1989). Melatonin is a potent inhibitor of dopamine release in the retina (Dubocovich, 1983), and is thought to have commensurate effects on circadian patterns of dopamine synthesis (Doyle et al., 2002a). Thus, the reduced levels of DACs function in C3H mice may be further exacerbated by the inhibiting effect of melatonin, while in C57 the function of persisting DACs is not suppressed by melatonin.

While further work is needed to determine the possible role of melatonin or other factors contributing to progression of RD and retinal remodeling, our findings suggest that the effect of genetic background on the health of DACs may directly influence the onset of retinal degeneration. This underscores both the importance of genetic background and the dopaminergic system in RD.

Functional implications of DAC dysfunction

Inner retinal neurons become spontaneously hyperactive during RD (Margolis et al., 2008; Stasheff, 2008; Ye and Goo, 2007), driven largely by rhythmic oscillations of AII amacrine cells (Borowska et al., 2011; Cembrowski et al., 2012; Choi et al., 2014; Margolis et al., 2014). DACs exhibit an opposite trend: in wt, many DACs have spontaneous rhythmic bursting activity, but become non-bursting in RD (Atkinson et al., 2013; Zhang et al., 2007).

How might this occur, particularly given the hyperactivity of ON cone bipolar cells, which provide direct excitatory input to DACs (Newkirk et al., 2013)? The reduced spontaneous activity suggests an increase of inhibitory synaptic input to DACs during RD, driven by hyperactive inhibitory neurons. DACs receive glycinergic and GABAergic inhibition from amacrine cells driven by rod bipolar cells and OFF cone bipolar cells, respectively (Newkirk et al., 2013). The degeneration of rod photoreceptors, the cells primarily affected in rd10, rd1 and rd1/C57 mice, can increase glycinergic inhibition of DACs by elevating membrane fluctuations of rod bipolar cells (Borowska et al., 2011). In turn, a reduction in DAC-mediated inhibition could lead to the activation of intrinsic oscillations in AII amacrine cells (Cembrowski et al., 2012; Choi et al., 2014). Tonic oscillations in AII cells may then transfer to OFF cone bipolar cells via a glycinergic synapse (Poria and Dhingra, 2014), increasing GABAergic inhibition of DACs (Atkinson et al., 2013), creating an inhibition-disinhibition loop. Thus, DACs may be integral to the generation of oscillatory hyperactivity. DACs also receive direct excitatory input from ON cone bipolar cells and intrinsically photosensitive retinal ganglion cells, but it appears that this input is not sufficient to overcome increased inhibition to restore bursting activity (Newkirk et al., 2013; Zhang et al., 2008).

The deficit in DACs during RD may also be responsible for the propagation of oscillatory hyperactivity, which may have implications for retinal health overall. In RD, synaptic hyperactivity propagates via gap junctions in AII amacrine cells, and can be reduced with gap junction blockers (Toychiev et al., 2013). This could be due to a lack of dopaminergic input, as dopamine is an important regulator of gap junction coupling (Witkovsky, 2004).

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Figure 1.

Analysis of the retina for specific features. Cell counts of DACs were performed in each of four quadrants (Ventral, Nasal, Dorsal, and Temporal; outlined in gray), denoted by the directional symbol (lower right). The temporal quadrant is marked by an incision (arrow, left). Soma sizes and primary dendrite densities were measured in areas marked by white boxes. Measurements of the axonal system were performed within the areas marked by black boxes. Scale bar, 1 mm.

Figure 2.

Distribution of DACs in wildtype and RD retinas. (A–D) DACs were immunolabeled for tyrosine hydroxylase. Insets show locations of the cell somas in red. Ventral, Nasal, Dorsal, and Temporal quadrants denoted by directional symbol; for *rd1C57* D pole is marked. Scale bar, 1 mm. (E) Total number of DACs in *rd1* retinas was significantly less than wt ($p < 0.0001$).

Figure 3.

Morphological changes of somas and primary dendrites of DACs vary across retinal quadrants in RD. Magnified areas from ventral (A–H) and dorsal (I–P) regions from the same retinas shown in Figure 2. Representative somas are shown in upper insets ($\times 2.52$ further magnification). Tracings of primary dendrites are shown with highlighted soma in lower insets. (Q) Soma size was reduced in RD ($p < 0.0001$). (R) Primary dendrite density was reduced in RD ($p < 0.0001$). The density of rd1 dendrites was also significantly less than rd10 ($p = 0.002$). Scale bar, 0.1 mm.

Figure 4.

Chromatin distribution, TH expression, and cytoplasm volume of DACs were different in RD. Single confocal images of DAC somas in the retinal wholemounts from wt, rd10, rd1 and rd1/C57 mice, aged P180. Retinas were double labeled for TO-PRO-3 (A–D, blue) and tyrosine hydroxylase (TH, E–H, red). All samples were taken from central part of the temporal quadrant. Scale bar, 5 μ m.

Figure 5.

Morphological changes within the axonal system of DACs. (A–L) Single confocal images of TH⁺ processes (cyan) in retinal wholemounts double-labeled for VMAT2 (green). Arrowheads point to the primary dendrites. In wt, the intensity of TH labeling was comparable between varicosities and slender connecting processes. In RD the TH-staining was concentrated in varicosities. (M) Reduction in TH⁺ particle size in rd10 and rd1 ($p < 0.0001$). (N) Increase in rd1 VMAT2 particle size ($p < 0.0001$). Scale bar 10 μm .

Figure 6.

Dopaminergic rings around AII amacrine cells are reduced in RD. (A–C) Viral construct for targeting expression of GFP in AII amacrine cells (top). Somas of GFP-labeled AII amacrine cells in retinal wholemounts. Inset shows a single reconstructed AII amacrine cell. (D–F) Dopaminergic rings are visible in TH+ processes (cyan) around AII somas labeled by GFP (green). Scale bars, 50 μ m in A, 10 μ m in B.

Figure 7.

Dopaminergic contacts to AII amacrine cells are reduced in RD. (A–F) Somas and lobular appendages of representative AII amacrine cells (green) with the adjacent TH+ processes (cyan). Note that arboreal dendrites of AII amacrine cells are not shown. (G) Surface area of TH+ contacts to AII soma measured are reduced in rd10 ($p = 0.002$) and rd1 ($p < 0.001$). Scale bar, 5 μm.

Combining immunolabeling for tyrosine hydroxylase to quantify dopaminergic cells (magenta) and AAV-mediated GFP-labeling of target AII amacrine cells (green) in the retina, the authors demonstrate deconstruction of the dopaminergic amacrine cell network during photoreceptor degeneration.

Table 1

Primary Antibodies

Antigen	Immunogen	Manufacturer, species antibody was raised in and type, catalog #	Dilution	Reference
Tyrosine hydroxylase	Denaturated tyrosine hydroxylase from rat pheochromocytoma	Millipore, purified rabbit polyclonal, Cat# AB152, RRID:AB_390204	1:10,000	Hendrickson et al., 1981
Vesicular monoamine transporter 2 (VMAT2)	Peptide from intracellular C-terminal region of human VMAT2 (C-TQNNIQSYPIGEDEESED-OH)	PhosphoSolutions, purified sheep polyclonal, Cat# 2200-VMAT2C, RRID:AB_2315595	1:10,000	Haycock et al., 2003; Witkovsky et al., 2004