Functional integrity and modification of retinal dopaminergic neurons in the rd1 mutant mouse: the roles of melanopsin and GABA

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ABSTRACT

The progressive loss of rod and cone photoreceptors in human subjects with retinitis pigmentosa causes a gradual decline in vision and can result in blindness. Current treatment strategies for the disease rely on the integrity of inner retinal neurons, such as amacrine cells, that are postsynaptic to photoreceptors. Previous work has demonstrated that a specialized subclass of retinal amacrine cell that synthesizes and releases the key neurotransmitter dopamine remains morphologically intact during the disease; however, the pathophysiological function of these neurons remains poorly understood. Here we examined spontaneous and light-evoked spike activity of genetically-labeled dopamine neurons from the retinas of retinal degeneration 1 (rd1) mice. Our results indicated that rd1 dopamine neurons remained functionally intact with preserved spontaneous spiking activity and light-evoked responses. The light responses were mediated exclusively by melanopsin phototransduction, not by surviving cones. Our data also suggested dopamine neurons were altered during photoreceptor loss, as evidenced by less spontaneous bursting activity and increased light-evoked responses with age. Further evidence showed that these alterations were attributed to enhanced GABA/melanopsin signaling to dopamine neurons during disease progression. Taken together, our studies provide valuable information regarding the preservation and functional modification of the retinal dopamine neuronal system in rd1; this information should be considered when designing treatment strategies for retinitis pigmentosa.

KEYWORDS

Dopamine; amacrine cell; melanopsin; GABA; retinitis pigmentosa.
INTRODUCTION

The vertebrate retina contains three unique classes of photosensitive cells: the outer-retinal rod and cone photoreceptors which are essential for image-forming vision, and the recently discovered inner-retinal intrinsically photosensitive retinal ganglion cells (ipRGCs) which are obligatory for non-image-forming visual responses (e.g. circadian photoentrainment and pupillary light reflex) (Berson 2003). As a result, the loss of rods and cones in human subjects with retinitis pigmentosa leads to poor vision and can even cause blindness (Heckenlively 1988). Currently there is no effective treatment for the disease. Promising strategies for vision restoration include photoreceptor cell transplantation, stem cell and gene therapy, and electrical and cell-type-specific optical stimulation (Acland et al. 2001; Bi et al. 2006; Pearson et al. 2012; Weiland et al. 2005). These approaches rely critically on the structural and functional integrity and stability of inner retinal neurons including horizontal cells, bipolar cells, amacrine cells, and ganglion cells. It is therefore imperative to understand how these neurons respond to rod and cone degeneration. Previous studies have attempted to evaluate the structure and function of these neurons during retinal degeneration with a focus on horizontal cells, bipolar cells, and ganglion cells (Chen et al. 2012; Damiani et al. 2012; Lin et al. 2009; Mazzoni et al. 2008; Puthussery et al. 2009; Stasheff 2008; Strettoi and Pignatelli 2000; Strettoi et al. 2002; Varela et al. 2003; Yee et al. 2012); yet, limited information is available for amacrine cells, the third-order retinal neurons that have diverse functions in visual information processing (Borowska et al. 2011; Strettoi et al. 2002; Trenholm et al. 2012).

A key subclass of retinal amacrine cell is the dopaminergic amacrine neuron. These cells are the sole source of retinal dopamine which plays vital roles in a variety of retinal functions (Witkovsky 2004). This molecule can serve as a light mediator that reconfigures retinal neural circuits in light adaptation as well act as a clock regulator that controls retinal circadian rhythm. However, levels of
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retinal dopamine are decreased in dystrophic retinas; the underlying mechanisms are still unclear (Doyle et al. 2002; Hankins and Ikeda 1994; Nir et al. 2000; Nir and Iuvone 1994; Vugler et al. 2007). This decline could be due to a reduced number of retinal dopamine neurons, low levels of dopamine synthesis, or an impairment of dopamine release from the cells. Previous studies have demonstrated that the former two possibilities were unlikely, leaving to question whether the latter is involved in the underlying mechanisms (Frucht et al. 1982; Hankins and Ikeda 1994; Kato et al. 1981; Strettoi et al. 2002). In addition, although rod and cone photoreceptors are degenerated in dystrophic retinas, light is still capable of increasing retinal dopamine release (Doyle et al. 2002; Vugler et al. 2007). Again, the phototransduction source and mechanisms are unclear. A direct strategy for addressing these crucial issues is to determine how individual dopamine neurons functionally respond to rod and cone loss.

Dopamine neurons are known to fire action potentials (spikes) which are thought to be the principal trigger for dopamine release (White 1996). The amount of dopamine released appears to be determined by the spike frequency and pattern (single-spike, random, or bursting) (Floresco et al. 2003; Gonon 1988; Puopolo et al. 2001). Our recent studies show that retinal dopamine neurons in mice exhibit a mixture of spontaneous single spikes and bursts (Gustincich et al. 1997; Zhang et al. 2007). The spontaneous activity of the neurons is negatively regulated by GABAergic inhibitory synaptic inputs in darkness and elevated by glutamatergic excitatory synaptic inputs upon light stimulation (Zhang et al. 2008; Zhang et al. 2007). Further evidence demonstrates that the light-evoked activity is mediated by rods and cones through bipolar cells as well as by the novel photopigment melanopsin that is expressed in a small population of retinal ganglion cells (Zhang et al. 2008; Zhang et al. 2007). This novel information obtained from wild-type (WT) mice has provided a basis for exploring the pathophysiology of dopamine neurons in diseased mice such as the retinal degeneration 1 (rd1) mouse—an animal model of retinitis pigmentosa. Our initial studies from rd1 mice have demonstrated that some dopamine neurons are still activated by light (Zhang et al. 2012; Zhang et al. 2008). Here we expand our
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previous studies by demonstrating that (1) the intrinsic activity of dopamine neurons is preserved with a
decrease in bursting in rd1 retinas, (2) burst reduction is mediated by enhanced GABA signaling, (3) the
preserved light responses of dopamine neurons are mediated exclusively by melanopsin, and (4) the
novel neural pathway from ipRGCs to dopamine neurons is enhanced in advanced retinal degeneration.

MATERIALS AND METHODS

Animals. C3H/HeJ mice homozygous for the Pde6b<sup>rd1</sup> mutation (rd1) were purchased from the Jackson
Laboratory. This rd1 mouse model carries a mutation in the β-subunit of the rod photoreceptor cGMP
phosphodiesterase-6 (PDE-6). The mutation is characterized by initial rod loss followed by secondary
cone death. Rod loss occurs rapidly with onset at postnatal day 8 (P8) and is nearly complete by P21. By
P90, virtually all outer photoreceptors have disappeared except for approximately 3% of cone somata in
the dorsal retina (Carter-Dawson et al. 1978).

We crossed the rd1 mice with our transgenic mice (on a C57BL/6J background) in which dopamine
neurons are genetically marked by red fluorescent protein (RFP) under control of the tyrosine
hydroxylase (TH) promoter (Zhang et al. 2004). Resulting F1 rd1 heterozygous (het) mice were
genotyped for the presence of RFP and the positive animals were further crossed with rd1 mice. Male
and female offspring from the F2 generation were genotyped for RFP and the rd1 mutation. From this
cross we obtained TH::RFP mice homozygous for the rd1 mutation (rd1TH::RFP) to use for
experiments. The rd1 het TH::RFP mice we obtained were used for control experiments. We examined
the gross anatomy of the living retinas isolated from rd1 and rd1 het TH::RFP transgenic mice (1-13
months old) and found that although these mice had a mixed C3H and C57BL/6J background, rd1
TH::RFP transgenic mice had the same phenotype as the rd1 mice, and the rd1 het TH::RFP transgenic
mice had the same phenotype as the WT TH::RFP transgenic mice.
In order to genetically knockout the photopigment melanopsin in rd1 mice, rd1 TH::RFP transgenic mice were further crossed with melanopsin knockout (opn4−/−) mice on a 129SV background (kindly provided by Dr. Samer Hattar at Johns Hopkins University) (Hattar et al. 2003). RFP-positive F1 male and female offspring were crossed to gain an F2 generation. The resulting F2 offspring were genotyped and the mice lacking opn4, homozygous for the rd1 mutation, and positive for RFP (opn4−/− rd1 TH::RFP transgenic mice) were used for experiments. Again, we found the anatomical structures of the living retinas from this line of mice (1-4 months old) had the same phenotype as the rd1 mice. All animals were maintained under 12 h light: 12 h dark conditions. All procedures conformed to NIH guidelines for work with laboratory animals and were approved by the Institutional Animal Care and Use Committee at Oakland University.

Electrophysiological recordings. To avoid a circadian effect, all experiments were performed during the day. Mice were dark adapted for 1-2 h prior to experiments then euthanized by CO2 overdose and cervical dislocation. Their eyes were enucleated and hemisected at the ora serrata under infrared illumination. The cornea and lens were removed in a Petri dish filled with oxygenated extracellular solution containing (in mM): 125 NaCl, 2.5 KCl, 1 MgSO4, 2 CaCl2, 1.25 NaH2PO3, 20 glucose, and 26 NaHCO4. The retina was separated from the sclera then placed either photoreceptor side down (rd1 het retinas) or ganglion cell side down (rd1 retinas) in a recording chamber mounted on the stage of an upright conventional fluorescence microscope (Axio Examiner, Zeiss, Oberkochen, Germany) within a light-tight Faraday cage. Oxygenated extracellular medium (pH 7.4 with 95% O2 and 5% CO2) continuously perfused the recording chamber at a rate of 2-3 ml/min and the superfusate was held at 32-34°C by a temperature control unit (TC-344A, Warner Instruments, CT).

The retina was maintained in darkness for ~1 h prior to recording. Cells and recording pipettes were viewed on a computer monitor coupled to a digital camera (AxioCam, Zeiss, Oberkochen,
Dopamine neurons in rd1 retinas (Germany) mounted on the microscope. TH::RFP-expressing cells were first identified by fluorescence microscopy using a rhodamine filter set with a brief ‘snap-shot’ of fluorescence excitation light (1-5 s). The identified cells and glass electrode were visualized using infrared differential interference contrast (IR-DIC) optics for patch-clamp recording. Experiments began 10-15 min after fluorescence was used to locate the cells, allowing the retinas to recover from photobleaching (caused by the brief fluorescence excitation light). The recovery may be incomplete during this short dark-adaptation period, so our experiments were likely performed in a partially dark-adapted state.

Patch-clamp recordings were made from the soma of RFP-labeled dopamine neurons using 4-7 MΩ electrodes and signals were amplified using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, California). The pipette solution for whole-cell current-clamp experiments contained (in mM): 125 K-gluconate, 10 KCl, 0.5 EGTA, and 10 HEPES adjusted to pH 7.3 with KOH. The pipette solution for loose-patch recordings contained 150 mM NaCl and 10 mM HEPES adjusted to pH 7.4 with NaOH. Data were acquired via a Digidata 1440A digitizer (Molecular Devices) and analyzed offline using Clampex 10 software (Molecular Devices).

All drugs used for the experiments were purchased from Sigma Aldrich (St. Louis, MO) and prepared as concentrated stock solutions that were diluted to working concentrations in the extracellular medium.

Light stimulation. Light stimuli were generated using light-emitting diode (LED) lamps with 375 nm, 470 nm, and 525 nm wavelengths (LED supply, Randolph, VT; and L.C. Corp, Brooklyn, NY). An LED controller (Mightex, Pleasanton, CA) was used to drive the LEDs and light intensity was adjusted by varying the driving current. Light intensity was measured at the surface of the retina using an optical power meter (units converted from µW/cm² to photons · s⁻¹ · cm⁻²; model 840-C, Newport, Irvine, CA).
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**Statistical analyses.** Data were analyzed using the Clampfit 10 and SigmaPlot 12 (Systat Software, Germany) software packages. Firing rates and bursting activity were measured from 90-s recordings of individual cells using the Event Detection program of the Clampfit 10 software. The coefficient of variation (CV), defined as the standard deviation of the inter-spike intervals (ISIs) divided by the mean of the ISIs, was used to describe the variation of ISIs in spike trains. Compared to conventional measures of variation, such as sample variance and standard deviation, the CV is scale insensitive. Distributions of ISIs with a CV < 1 were considered to have low variance, whereas those with a CV ≥ 1 were considered to have high variance.

Bursts were detected by the “Poisson surprise” method (Legendy and Salcman 1985). It was assumed that spikes in a certain interval followed a Poisson distribution. This method calculated the probability ($P$) that a given spike train would be found. A group of spikes ($n ≥ 3$) was initially identified as a burst if the burst onset initiated by two consecutive spikes with an inter-spike interval < 80 ms and terminated with two spikes having an inter-spike interval > 160 ms (Grace and Bunney 1984). Bursts were assigned a Poisson surprise value as a quantitative measure of burst strength defined by: $-\log_{10}P$ (Legendy and Salcman 1985). Following the initial calculation of the Poisson surprise value, this value was maximized by adding additional intervals after the initial interval or deleting first intervals from the initial interval. The spike train was judged to be a burst if the Poisson surprise value was > 1.5, meaning that the particular group of spikes had less than a 1 in 32 chance of being a random event.

For peri-stimulus time histograms (PSTHs), light stimuli were presented once every 2 minutes and each PSTH was a summation of 3 to 5 stimulus presentations using 0.3 s bins. To calculate latency to peak spiking frequency, the distribution of spiking frequency during light responses was fit with a peak function; the peak time of the fitted function relative to stimulus onset was taken as the latency to peak spiking frequency. The distribution of spiking frequency of dopamine neurons from young mice (Fig. 7A) had a symmetrical peak shape which was best fit with a Lorentzian function, whereas the
Dopamine neurons in *rd1* retinas distribution from old mice (Fig. 7B) had a rapid onset, sharp peak, and slow decline which was best fit with a Weibull function.

We used one-way ANOVA to determine statistical significance of the firing properties across three independent groups of data. Comparison between two independent groups was made by a Student’s t-test when the data followed a normal distribution. When the distribution was not normal, a non-parametric Mann-Whitney U test was used. To assess the effects of drugs, we determined the significance of the firing properties before and during drug application using a paired t-test when the data had a normal distribution or a Wilcoxon signed-rank test if the data were not normally distributed.

In addition, proportion comparisons were accomplished using a two-tailed Fisher’s exact test. Values of the normally distributed data are given as the mean ± SEM; we have presented non-normally distributed data as a scatter plot along the y-axis with the mean indicated. The levels of significance were set at *P* < 0.05 (*), *P* < 0.01 (**), and *P* < 0.001 (***)

**RESULTS**

We first examined RFP expression in whole-mount living retinas isolated from *rd1 TH::RFP* and *opn4<sup>−/− rd1 TH::RFP* transgenic mice and found that in each retina, 5-15 cells were clearly marked with RFP. Fig. 1A displays an RFP-marked cell from an *rd1 TH::RFP* retina. Both the cell body and primary dendrites were strongly labeled by RFP which allows the cells to be visualized and targeted for recording. We verified that these RFP-labeled cells were indeed dopamine neurons by performing immunostaining with a TH antibody. It was found that the RFP-labeled cells in both *rd1 TH::RFP* and *opn4<sup>−/− rd1 TH::RFP* retinas were positive for TH (data not shown), suggesting that they are dopamine neurons, not type 2 catecholaminergic amacrine cells (Zhang et al. 2004). In addition, we examined RFP expression in *rd1 het TH::RFP* retinas in which photoreceptors remain intact; the number of RFP-
Dopamine neurons in rd1 retinas expressing cells and RFP intensity within the cells were indistinguishable from what we observed in WT TH::RFP retinas (Zhang et al. 2004). The exact reason for the disappearance of RFP in most rd1 dopamine cells is unclear, but the average of 10 RFP-expressing cells randomly distributed throughout each rd1 retina was sufficient for us to perform patch-clamp recording experiments.

Dopamine neurons in rd1 retinas fire dominantly single spontaneous spikes

Dopamine neurons respond to intrinsic and extrinsic factors to modulate spike frequency and pattern which controls the amount of dopamine release (White 1996). We therefore determined whether these critical firing properties were preserved or modified with the loss of rod and cone photoreceptors. RFP-expressing dopamine neurons were recorded in whole-mount retinas from rd1 and rd1 het mice using a loose-patch clamp technique. We found that the neurons recorded from retinas of both mice were spontaneously active. Although they had nearly identical firing rates, the firing patterns were clearly different.

In a total of 23 rd1 dopamine cells recorded, 6 of them fired in a pacemaker-like single-spiking pattern; Fig. 1B illustrates a typical example (left trace). The rest of the cells exhibited additional sparse clusters of spontaneous spikes (bursting) as shown in Fig. 1B (right trace). The value of the coefficient of variation (CV) of ISIs, a commonly used parameter for measuring dispersion from a probability distribution, was calculated for each cell. The distribution of the CV values from 23 cells is illustrated in Fig. 1D, ranging 0.08 to 1.82 with 96% of the values <1, indicating most cells had relatively low ISI variance.

In contrast, the CV values for rd1 het dopamine neurons ranged from 0.46 to 4.1; 63% of them were >1, indicating most cells had high variance of ISIs (Fig. 1D; n = 19). Fig. 1C shows two representative traces recorded from rd1 het dopamine neurons: one which had a low CV value (left) and
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the other which had a high CV value (right). Comparison of the CV values between rd1 het and rd1
dopamine neurons shows a significant difference (Fig. 1D; \( P < 0.001 \), Mann-Whitney U test), suggesting
that spontaneous bursting activity of dopamine neurons is significantly decreased in rd1 retinas. This
observed reduction was conserved from young to old rd1 mice (1 to 13 months); mean CV values of rd1
dopamine cells obtained from mice in age groups: 1-2, 6-7, and 12-13 months show no significant
difference between subjects (\( P > 0.05 \), one-way ANOVA, data not shown). In addition, the spiking rate
of dopamine neurons in rd1 het retinas ranged from 2.2 Hz to 14 Hz with a median of 5.3 Hz (n=19),
whereas it ranged from 1.5 Hz to 8.8 Hz with a median of 5.8 Hz (n=23) in rd1 dopamine neurons; no
significant difference was observed between these two groups (Figure 1E; \( P > 0.05 \), Mann-Whitney U
test).

The above data demonstrated that ~74% of rd1 dopamine neurons (17/23 cells) preserved their
bursting activity to some degree; it was therefore necessary to further characterize the bursting
properties of these cells and compare them to rd1 het dopamine neurons. Results showed contrasting
bursting properties of rd1 (n = 17) and rd1 het (n=19) dopamine cells with rd1 cells having fewer bursts
per second (bursting frequency, Fig. 2A; \( P < 0.01 \), Mann-Whitney U test), longer intra-burst inter-spike
intervals (intra-burst ISI, Fig. 2B; \( P < 0.05 \), Mann-Whitney U test), and a drastically lower percentage of
spikes within bursts (Fig. 2C; \( P < 0.001 \), Mann-Whitney U test). In addition, burst duration tended to
decrease in rd1 dopamine neurons, however, this decrease was not statistically significant (Fig. 2D; \( P >
0.05 \), Mann-Whitney U test). Collectively, our data further indicate that dopamine cell bursting activity
is substantially reduced in rd1 dopamine neurons compared to rd1 het dopamine neurons.

It is notable that the bursting properties of dopamine neurons in rd1 het retinas were quite
similar to those observed in WT TH::RFP retinas (Zhang et al. 2007); this finding suggests
heterozygosity for rd1, without significant changes in the number of photoreceptors, does not
significantly alter dopamine neuron bursting activity. Conversely, homozygosity for rd1, associated with
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photoreceptor loss, remarkably reduces dopamine neuron bursting activity suggesting that this reduction
is likely a result of photoreceptor loss. Since the bursting pattern of firing is predicted to result in more
efficient dopamine release at target loci, less bursting in rd1 dopamine neurons may account for the low
levels of basal dopamine previously reported in animal models of retinitis pigmentosa (Floresco et al.

GABA_A receptor blockade restores dopamine neuron bursting activity in rd1 retinas

We next sought to determine the underlying mechanisms responsible for the reduction in rd1
dopamine cell bursts. We have previously demonstrated that GABAergic input to dopamine neurons
inhibited their bursting activity in WT retinas (Zhang et al. 2007). If GABAergic signaling increases in
rd1 retinas, then this increase could reduce dopamine neuron bursting activity. To test this possibility,
GABA_A receptor antagonists (GABAzine or bicuculline) were administered to the retinas. Fig. 3A
displays representative traces before and during bath application of 20 µM GABAzine using loose-patch
extracellular recording. It is clearly shown that GABAzine drastically increased the bursting activity;
this increase was reversed upon washout (data not shown). Data analysis demonstrates that the CV value
was increased from 0.86 ± 0.12 in control to 1.48 ± 0.19 in the presence of GABAzine (Fig. 3B; n = 15;
P < 0.05, paired t-test), whereas the mean firing rate was increased from 5.01 ± 0.38 Hz to 8.26 ± 0.75
Hz (Fig. 3C; n = 15; P < 0.001, paired t-test). Similar results were obtained with bicuculline in whole-
cell current-clamp mode. As shown in Fig. 3D (left), the resting membrane potential of the cell was -55
mV and it was accompanied by dominant single action potentials. 100 µM bicuculline depolarized the
cell and produced oscillatory potentials that were crowned by bursts of action potentials (Fig. 3D, right).
Similar results were observed in 2 additional cells.
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We further analyzed the bursting properties of rd1 dopamine neurons before and during application of GABAzine in detail. It was found that GABAzine significantly increased bursting frequency (Fig. 4A; 0.16 ± 0.03 in control vs. 0.35 ± 0.04 in GABAzine; n = 12; P < 0.001, paired t-test), the percentage of spikes within bursts (Fig. 4B; 19.8 ± 3.0% in control vs. 59.6 ± 7.3% during GABAzine; n = 12; P < 0.001, paired t-test), and burst duration (Fig. 4C; n = 12; P < 0.001, Wilcoxon signed-tank test). GABAzine also significantly decreased the intra-burst ISIs (Fig. 4D; n=12; P < 0.01, Wilcoxon signed-rank test). It is worth noting that these effects are quite distinct from what we previously observed in WT dopamine neurons (Zhang et al. 2007). In the WT, GABAzine significantly increased only burst duration, indicating GABAzine had a much stronger effect on dopamine neurons in rd1 retinas than in WT retinas. This profound difference suggests that inhibitory GABAergic signaling to dopamine neurons is substantially enhanced in the rd1 retina. This enhanced signaling appears to hyperpolarize the cells which could result in a decrease of bursting activity.

Light-evoked responses remain intact in some rd1 dopamine neurons

It was intriguing to observe that some dopamine neurons still responded to light although all rods and most cones are degenerated in rd1 retinas (Zhang et al. 2012; Zhang et al. 2008); here we wanted to determine more detailed information regarding this observation. We tested light responses from dopamine neurons in rd1 retinas of mice ranging from 1 to 13 months old. Each cell was subjected to various wavelengths of light (375 nm for ultraviolet cone opsin; 470 nm for melanopsin; and 525 nm for rod and middle-wavelength sensitive cone opsins) as well as low and high light intensities (1.26 × 10^{12} and 2.25 × 10^{13} photons · s^{-1} · cm^{-2}, respectively). Fig. 5A shows a loose-patch recording of one cell having a rapid increase in firing frequency to a 470-nm light pulse (3-s duration, 1.26 × 10^{12} photons · s^{-1} · cm^{-2}). We also conducted whole-cell current-clamp recordings and found that the light-evoked spiking
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increases in dopamine neurons were associated with the membrane depolarization of the cells to light (Fig. 5B). This class of light response was observed from young to old mice although response dynamics were quite distinct with age (see below). Of a total of 33 cells tested, 30 of them (~91%) exhibited an increased spike frequency upon light stimulation which in most cases persisted during light stimulation. The other 3 cells did not show responses to 470 nm light (Fig. 5C) or the other wavelengths tested (data not shown).

We should point out that the percentage of light responsive dopamine neurons (~91%) obtained in rd1 retinas is not comparable to that reported previously in WT retinas (~60%) (Zhang et al. 2007). This is because, as described above, only approximately 2% of dopamine cells (~10 dopamine cells per retina) were labeled by RFP in rd1 retinas and the recordings were made from this small proportion of the total cells. It is unclear whether the other 98% of dopamine neurons are light responsive. In WT retinas, 89% of dopamine neurons (~400 dopamine cells per retina) were labeled by RFP and the recordings were randomly taken from this large proportion of the cells (Zhang et al. 2004); ~60% of those cells (~240 cells per retina) were light responsive while the rest were resistant to light (Zhang et al. 2007). Although we previously suggested that dopamine neuron light responses in rd1 retinas are mediated by melanopsin in ipRGCs (Zhang et al. 2012; Zhang et al. 2008), we had not yet ruled out the possibility that these light responses may be mediated by light activation of surviving cones or other unidentified photosensitive cells in the inner retina.

Melanopsin knockout eliminates light-evoked responses of dopamine neurons in rd1 retinas

To test whether the remaining light responses of rd1 dopamine neurons were mediated by the photopigment melanopsin, we genetically knocked-out this light-absorbing molecule in rd1 TH::RFP transgenic mice (opn4−/− rd1 TH::RFP mice) and reexamined dopamine neuron light responses in these
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subjects. We started with 1 to 2-month-old animals in which the cell bodies of some cone cells are still present in the retina (Lin et al. 2009). It was found that none of the 13 dopamine neurons recorded were sensitive to 375, 470, or 525-nm light (light intensities: $1.26 \times 10^{12}$ or $2.25 \times 10^{13}$ photons \cdot s^{-1} \cdot cm^{-2}$). Fig. 6 (A-C) displays one such example using these three wavelengths at an intensity of $1.26 \times 10^{12}$ photons \cdot s^{-1} \cdot cm^{-2}$. The same results were observed in 6 cells recorded from the retinas of 3 to 4 month-old mice (data not shown). We further compared the proportion of light responsive cells in *rd1* retinas with and without melanopsin (30/33 in *rd1* retinas vs. 0/19 in *opn4*⁻/⁻ *rd1* retinas; Fig. 6D); the difference between them is statistically significant ($P < 0.001$, two-tailed Fisher’s exact test). These results clearly indicate that dopamine neuron light responses in *rd1* retinas are mediated by melanopsin phototransduction and not by surviving cones.

Melanopsin-mediated light responses of dopamine neurons increase from young to old *rd1* animals

The structural and functional composition of retinal synaptic circuitry generally undergoes extensive remodeling during retinal degeneration, particularly during phase 3 (phases 1 and 2 are rod degeneration and cone degeneration, respectively) (Marc et al. 2007; Marc et al. 2003; Vugler 2010). We therefore hypothesized that the novel neural pathway from ipRGCs to dopamine neurons is functionally modified from phase 2 to phase 3. To test this hypothesis, we measured light-evoked responses of dopamine neurons from the retinas of *rd1* mice at ages 1 to 2 months old (phase 2) and 6 to 7 months old (phase 3) and then compared the age-group characteristics. It appears that the responses from younger mice (Fig. 7A, *left*) peaked more slowly than older mice (Fig. 7B, *left*). The peri-stimulus time histogram was successfully fit with a Lorentzian distribution for dopamine neurons recorded in younger mice (Fig. 7A, *right*) and a Weibull distribution for the recordings collected from older mice (Fig. 7B, *right*). From the fitted curves we measured the peak latency (time from light onset to peak) for
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each response and found that the peak latency significantly decreased from 1.95 ± 0.12 s (n = 5) in younger mice to 0.94 ± 0.14 s (n = 5) in older mice (P < 0.001, Student’s t-test). In contrast, the peak firing frequency of the responses was much lower in younger mice than in older mice. The firing rate peak amplitudes (from curve: mean spontaneous firing rate subtracted from the peak firing rate) significantly increased from 9.7 Hz in younger mice (n = 5) to 85.8 Hz in older mice (n = 5; P < 0.05, Mann-Whitney U test). These results suggest that the strength of melanopsin-driven signaling to dopamine neurons increases from phase 2 to phase 3 of retinal degeneration.

DISCUSSION

In this study, we have taken advantage of our transgenic mouse line in which dopamine neurons are marked by RFP under control of the TH promoter in combination with rd1 mutant mice in which rods rapidly degenerate soon after birth. Using this genetic strategy we have been able to visualize dopamine neurons and target them for recording in the diseased mice. The major findings of our study are: (1) dopamine neurons in rd1 retinas retain their spontaneous spiking activity with a decrease in bursting; (2) burst reduction is mediated by enhanced GABA signaling; (3) light responses of rd1 dopamine neurons are mediated exclusively by melanopsin photoreception and not by surviving cones; and (4) the retrograde signaling pathway from ipRGCs to dopamine neurons is enhanced in advanced retinal degeneration.

Physiological properties of dopamine neurons are partially preserved in the absence of rods and cones

Our results show that dopamine neurons in the rd1 retina, like in the WT, fire spontaneously; this finding indicates that these diseased dopamine neurons have the ability to generate the action potentials
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required for dopamine release. In addition, dopamine neuron activity can also be regulated by external factors (e.g. light), suggesting that these cells continue to communicate with other retinal cells involved in regulating dopamine release. Furthermore, previous reports have indicated that dopamine neuron morphology is not significantly altered by the loss of rods and cones (Hankins and Ikeda 1994; Strettoi et al. 2002). Taken together, the overall results suggest that dopamine neuron structure and function is at least partially preserved in rd1 retinas. Additionally, retinal ganglion cells, another type of third-order retinal neuron, also show this preserved structure and function in rd1 retinas (Margolis et al. 2008; Mazzoni et al. 2008; Stasheff 2008; Ye and Goo 2007). Because some vision-restorative treatment strategies for retinal degeneration (e.g. photoreceptor cell transplantation) require donated cells to make connections with existing retinal circuitry and inner retinal neurons, the preservation of the third-order retinal neurons may be important for these therapies.

Reduction of dopamine neuron spontaneous bursting activity in rd1 retinas is mediated by GABA

Although rd1 dopamine neurons retain a normal firing rate, our data show that their firing patterns are altered. The cells were observed to fire either in an irregular single-spiking pattern or with sparse bursts. This is in contrast to rd1 het dopamine neurons which exhibit a relatively high bursting frequency, high percentage of spikes within bursts, and short intra-burst inter-spike intervals. Considering that both the rd1 and the rd1 het mice were of the same genetic background, this distinction cannot be attributed to background difference.

Our results demonstrate that the loss of rods and cones affects the GABAergic system to alter dopamine neuron spontaneous firing pattern. This is shown through the observation that GABA\textsubscript{A} receptor antagonists depolarize rd1 dopamine neurons as well as restore their loss of bursts. The simplest interpretation of this effect is that GABA content increases during retinal degeneration
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(Murashima et al. 1990); however, we cannot rule out molecular alterations of GABA receptors on rd1 dopamine neurons which could be causing the increased responsiveness to GABA (Yazulla et al. 1997). These possibilities include, but are not limited to: changes in receptor sensitivity, the number of receptors, receptor subunit composition, and endogenous ligands of auxiliary sites such as benzodiazepine (Wong et al. 2003).

Interestingly, the firing pattern changes seen in rd1 dopamine neurons are completely opposite of rd1 retinal ganglion cells which display hyperactivity and rhythmic bursting (Margolis et al. 2008; Stasheff 2008; Ye and Goo 2007; Yee et al. 2012). Recent reports suggest that intrinsic oscillatory potentials were induced in bipolar cells (and AII amacrine cells) of rd1 retinas (Borowska et al. 2011; Menzler and Zeck 2011), thus providing the presynaptic mechanism responsible for retinal ganglion cell burst generation. If this were the case for dopamine neurons, these neurons would also have increased bursting activity; however, we observed the opposite effect that dopamine neuron bursting activity was decreased. It is evident that the loss of rods and cones alters dopamine neurons and retinal ganglion cells through distinctly different mechanisms; these mechanisms should be carefully considered when designing treatment strategies for retinitis pigmentosa.

Melanopsin mediates light-induced responses of dopamine neurons in rd1 retinas

In our previous studies we have found that some of dopamine neurons were responsive to light in the rd1 retina (Zhang et al. 2008). The present study provides evidence that these light responses are mediated exclusively by melanopsin phototransduction; this is evident because of the complete absence of a light-evoked response in opn4c/- rd1 retinas. This result is consistent with our recent report that normal retinas lacking melanopsin only exhibited rod- and cone-driven transient responses (Zhang et al. 2012). It is therefore conceivable that melanopsin mediates retrograde signaling from ipRGCs to
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dopamine neurons in normal retinas and that this signaling is well-preserved in hereditary retinal
dystrophies.

Our study likely excludes the involvement of surviving cones in driving dopamine neuron
activity in rd1 retinas. The youngest opn4+/− rd1 mouse used was 42 days old, an age at which a
significant number of cones are still present in the retina; however, we were unable to obtain any light
responses from these dopamine neurons using various light intensities and wavelengths. This finding is
not surprising considering previous studies have shown that cones surviving for extended periods of
time in rd1 retinas appear incapable of sending light-driven information through the retina to mediate
visual or non-visual functions (Panda et al. 2003; Pearson et al. 2012; Provencio et al. 1994; Strettoi et
al. 2002). Our results also suggest that other inner retinal photoreceptive sources, such as paradoxical
opsin or UV-sensitive Opn5 (Kojima et al. 2011; Semo et al. 2007), are unlikely to have any
involvement in mediating light regulation of dopamine neuron activity.

Retrograde melanopsin signaling to dopamine neurons is enhanced in advanced retinal degeneration

We compared light-evoked responses of dopamine neurons between young and old rd1 mice and
found that signal transmission from ipRGCs to dopamine neurons is increased with age. The increase is
unlikely to be caused by aging because such phenomena was not seen in our preliminary studies using
the same ages of young and old WT mice (data not shown). Alternatively, this increase appears to be a
result of retinal remodeling occurring in advanced retinal degeneration. At this point it is unclear which
underlying mechanisms are involved in this process but it is unlikely that this alteration is due to
changes in ipRGCs, the presynaptic photosensitive neurons. This argument is strengthened by the
observation that the number of ipRGCs does not increase and that melanopsin expression in individual
cells remains unchanged in rd1 retinas (Vugler et al. 2008). We therefore speculate that the ipRGC-
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dopamine neuron neural circuit undergoes structural rewiring during progressive retinal degeneration which increases the number of contacts between dopamine neurons and ipRGCs. Other possibilities could be that during retinal degeneration glutamate released from ipRGCs increases and/or there are an increased number of glutamate receptors on dopamine neurons which could also result in enhanced melanopsin signaling to dopamine neurons. Future studies are needed to test these possibilities.

The retrograde melanopsin-signaling pathway to dopamine neurons is newly discovered and its function remains fully established in normal and diseased retinas. Without rods and most cones in the *rdl* retina, melanopsin is the only functional photopigment that is expected to drive dopamine release. Since there is a limited number of dopamine neurons driven by melanopsin signaling, the amount of retinal dopamine may be undetectable by high performance liquid chromatography (a method that measures tissue dopamine content and does not detect the functional pool of dopamine) (Cameron et al. 2009); a future study will certainly need to utilize a highly sensitive carbon fiber electrode to detect the dopamine released from dopamine neurons *in situ* (Puopolo et al. 2001).

In summary, we have shown that dopamine neuron spontaneous activity is altered in the disease with less bursting which could result in a decreased release of dopamine. This answers the long-standing question as to why dopamine release is decreased although the number of dopamine neurons and dopamine synthesis remains unchanged in retinal degeneration (Hankins and Ikeda 1994; Kato et al. 1981; Nir and Iuvone 1994). A similar mechanism may also apply to other retinal neurodegenerative diseases, such as diabetic retinopathy, in which the dopaminergic system is impaired (Nishimura and Kuriyama 1985). In addition, our results indicate that melanopsin is the photopigment mediating light regulation of dopamine neurons in *rdl* mice. This provides a molecular mechanism by which light regulates the retinal circadian clock through the release of dopamine in the absence of rods and cones (Doyle et al. 2002; Ribelayga et al. 2008; Ruan et al. 2008; Vugler et al. 2007). Furthermore, dopamine can act via D1-family receptors on ipRGCs through a feedback mechanism which may shape the
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responses of ipRGCs and thus of non-image-forming visual functions including the pupillary light reflex
and circadian photoentrainment (Panda et al. 2003; Van Hook et al. 2012).

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: C.L.A., J.F., and D.-Q.Z. conceived and designed the experiments; C.L.A., J.F.,
and D.-Q.Z. performed the experiments; C.L.A., J.F., and D.-Q.Z. contributed reagents/materials/analysis tools; C.L.A. and D.-Q.Z. analyzed the data; C.L.A and D.-Q.Z. interpreted results of the experiments; D.-Q.Z. drafted the manuscript; C.L.A. and D.-Q.Z. prepared the figures;
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C.L.A. and D.-Q.Z. edited and revised the manuscript; C.L.A, J.F., and D.-Q.Z. approved the final version of the manuscript.

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**FIGURE LEGENDS**

Fig. 1. Dopamine neurons in rd1 retinas fire dominantly in a single-spiking pattern. A: An RFP-labeled dopamine neuron imaged from a whole-mount rd1 retina; an arrowhead and arrow indicate a cell body and a primary dendrite, respectively. Loose-patch recordings were performed from rd1 and rd1 het dopamine neurons. The pipette holding potential was set at 0 mV. Spontaneous pacemaker-like spiking activity was observed in some rd1 neurons (B, left trace) and additional sparse, short-duration bursts were seen in other neurons (B, right trace). In contrast, rd1 het dopamine neurons exhibited a mixture of single spikes and bursts (C, left trace) and some of them fired dominantly in a bursting pattern (C, right trace). The mean coefficient of variation was greatly reduced in rd1 neurons (23 neurons collected from 15 retinas) compared to rd1 het controls (19 neurons collected from 13 retinas) (D; ***P < 0.001). In contrast, mean spiking rate between rd1 (n = 23) and rd1 het dopamine cells (n = 19) showed no significant difference (D; P > 0.05). For D and E, line segments indicate data means.

Fig. 2. Burst properties of dopamine cells are altered in rd1. Bursting properties of 17 rd1 neurons and 19 rd1 het neurons were analyzed (see Methods). Mann-Whitney rank sum tests were used to determine significant differences between the bursting properties of rd1 and rd1 het cells. The data showed that the rd1 dopamine cells had a significantly reduced bursting frequency (A; **P < 0.01), longer intra-burst ISIs (B; *P < 0.05), and a large reduction in the percentage of spikes within bursts (C; ***P < 0.001). Burst duration was reduced in rd1 but was not statistically significant (D; P > 0.05). Line segments indicate data means.

Fig. 3. Blockade of GABA<sub>A</sub> receptors with GABAzine or bicuculline restores bursting activity lost in rd1. A: a tracing of spontaneous spiking activity of an rd1 dopamine neuron in the absence (left panel) and presence (right panel) of 20 µM GABAzine shows GABAzine dramatically increased bursting activity. B: GABAzine significantly increased the mean CV (*P < 0.05; n = 15) and firing frequency (C;
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***P < 0.001; n = 15). 15 neurons were used from 7 retinas. D: Whole-cell current-clamp recordings were performed on rd1 dopamine neurons with the holding current set to 0 pA. A neuron had spontaneously generated rhythmic spikes at the resting membrane potential of -55 mV (left trace). Application of bicuculline to this cell resulted in oscillatory membrane depolarization and rhythmic bursting spikes (right trace). An arrow points to an oscillatory potential. Dashed line: -55 mV.

Fig. 4. Effects of GABA\textsubscript{z}ine on rd1 dopamine neuron burst properties. Burst properties of 12 neurons were analyzed and compared before and during application of 20 \(\mu\text{M}\) GABA\textsubscript{z}ine. GABA\textsubscript{z}ine significantly increased burst frequency (A; ***P < 0.001), the percentage of spikes within bursts (B; ***P < 0.001), and burst duration (C; ***P < 0.001). GABA\textsubscript{z}ine also caused a decrease in the intra-burst ISI (D; **P < 0.01). For C and D, line segments indicate data means.

Fig. 5. A population of rd1 dopamine neurons remains light-excitable. Loose-patch recording shows an example of an rd1 dopamine neuron that had increased spiking activity to a 470-nm light pulse (3-s duration, \(1.26 \times 10^{12}\) photons \(\cdot \text{s}^{-1} \cdot \text{cm}^{-2}\)) which persisted during light (A). Whole-cell current-clamp recording further shows that the same 470-nm light substantially depolarized an rd1 dopamine neuron with increased spiking activity (B). Other dopamine neurons were not light-excitable (C). Lights-on is indicated by a step-up on the line beneath the traces.

Fig. 6. Dopamine neuron light responses are completely ablated in melanopsin knock-out rd1 mice.

Light-induced activity of dopamine neurons in an opn4\textsuperscript{–/–} rd1 retina was tested at 375 nm (A), 470 nm (B), and 525 nm (C) wavelengths of light in the same cell under the same stimulation duration (3 s) and intensity (\(1.26 \times 10^{12}\) photons \(\cdot \text{s}^{-1} \cdot \text{cm}^{-2}\)). For A-C, original traces of spontaneous activity are shown in the left panels and the corresponding peri-stimulus time histograms (PSTHs) constructed with 3 stimulus presentations using 0.3-s bins are shown in the right panels. Both the original traces and PSTHs show no increase in spiking activity to any light stimulus. The same results were observed in 18 additional
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neurons tested, in a total of 6 retinas. The proportion of light responsive cells in opn4−/− rd1 retinas (0/19) is significantly different from rd1 retinas where ~91% of dopamine neurons (30/33) exhibited responses to light (D; ***P < 0.001). Lights-on is indicated by a step-up on the line beneath the traces or by a white box above the PSTHs.

Fig. 7. Light-evoked responses of dopamine neurons in rd1 mice increase with age. Light-evoked spike activity of dopamine neurons recorded from retinas of postnatal day 42 (P42) (A) and P182 (B) mice are compared. The left panels show original traces of activity and the right panels are their corresponding PSTHs constructed with 3 stimulus presentations using 0.3-ms bins. The PSTH for the P42 cell was best fit with a Lorentzian distribution curve, whereas the response curve of the P182 cell was best fit with a Weibull distribution. The young cell (P42) had a slower time to peak and a weaker sustained response, while the old cell (P182) responded more quickly to light and had a more pronounced sustained response during light and after lights-off. Lights-on is indicated by a step-up on the line beneath the traces or by a white box above the PSTHs. Light stimuli: 470 nm; light intensity: 1.26 × 10^{12} photons · s^{−1} · cm^{−2}; 3-s duration.
Fig. 4

(A) Bursting Frequency (Hz)

(B) Spikes within Bursts (%)

(C) Burst Duration (ms)

(D) Intra-burst ISI (ms)
A  Responsive

![Graph A](image)

B  Responsive

![Graph B](image)

C  Unresponsive

![Graph C](image)