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Early diabetes impairs ON sustained ganglion cell light responses and adaptation without cell death or dopamine insensitivity

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Abstract

Retinal signaling under dark-adapted conditions is perturbed during early diabetes. Additionally, dopamine, the main neuromodulator of retinal light adaptation, is diminished in diabetic retinas. However, it is not known if this dopamine deficiency changes how the retina responds to increased light or dopamine. Here we determine whether light adaptation is impaired in the diabetic retina, and investigate potential mechanism(s) of impairment. Diabetes was induced in C57BL/6J male mice via 3 intraperitoneal injections of streptozotocin (75mg/kg) and confirmed by blood glucose levels more than 200 mg/dL. After 6 weeks, whole-cell recordings of light-evoked and spontaneous inhibitory postsynaptic currents (IPSCs) or excitatory postsynaptic currents (EPSCs) were made from rod bipolar cells and ON sustained ganglion cells, respectively. Light responses were recorded before and after D1 receptor (D1R) activation (SKF-38393, 20 μ M) or light adaptation (background of 950 photons $\cdot \mu\text{m}^{-2} \cdot \text{s}^{-1}$). Retinal whole mounts were stained for either tyrosine hydroxylase and activated caspase-3 or GAD65/67, GlyT1 and RBPMS and imaged. D1R activation and light adaptation both decreased inhibition, but the disinhibition was not different between control and diabetic rod bipolar cells. However, diabetic ganglion cell light-evoked EPSCs were increased in the dark and showed reduced light adaptation. No differences were found in light adaptation of spontaneous EPSC parameters, suggesting upstream changes. No changes in cell density were found for dopaminergic, glycinergic or GABAergic amacrine cells, or ganglion cells. Thus, in early diabetes, ON sustained ganglion cells receive excessive excitation under dark- and light-adapted conditions. Our results show that this is not attributable to loss in number or dopamine sensitivity of inhibitory amacrine cells or loss of dopaminergic amacrine cells.

Keywords

diabetes; retina; inhibition; bipolar cell; ganglion cell; dopamine; light adaptation

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1.1 Introduction:

As of 2016 diabetes mellitus affected an estimated 422 million adults globally (N.C.D. Risk Factor Collaboration, 2016). As ~35% of those afflicted with diabetes mellitus eventually develop some form of diabetic retinopathy (DR), and ~10% develop a vision-threatening retinopathy (Yau et al., 2012), the associated vision loss represents a highly significant challenge to global health. It is now well established that an early consequence of diabetes is a deficit in retinal neuronal function, prior to a typical clinical diagnosis of DR (Barber and Baccouche, 2017; Garcia-Martin et al., 2019; Gundogan et al., 2016; Kizawa et al., 2006; Pardue et al., 2014; Simo et al., 2018). This deficit can be measured via electroretinogram (ERG), which represents the net electrical activity of retinal neurons in response to light stimuli. Previous studies have shown changes in scotopic (dim light) ERG response latency, b-wave amplitude, and oscillatory potentials in diabetic patient populations that have yet to develop DR (Holopigian et al., 1992; Juen and Kieselbach, 1990; Kawasaki et al., 1986; Luu et al., 2010), suggesting changes in rod bipolar cell and amacrine cell activity (Pinto et al., 2007; Wachtmeister, 1998). These early changes are largely mirrored in animal models of diabetes (Aung et al., 2013; Bui et al., 2009; Kohzaki et al., 2008; Pardue et al., 2014; Ramsey et al., 2006). Importantly, some studies suggest that these early neuronal changes may be involved in the progression of DR (Fortune et al., 1999; Han et al., 2004; Harrison et al., 2011; Ng et al., 2008).

There is growing evidence that retinal dopaminergic signaling is also affected during early diabetes (Lahouaoui et al., 2016; Tian et al., 2015; Vancura et al., 2016), and may underlie these early neuronal deficits as observed via ERG (Aung et al., 2014; Kim et al., 2018). Dopamine mediates retinal light adaptation, the process that changes retinal sensitivity to allow us to see under both very bright and dim conditions, which is thus crucial to normal visual function (Jackson et al., 2012; Roy and Field, 2019; Witkovsky, 2004). It is currently unknown whether this early dopaminergic disruption in the diabetic retina is attributable to changes in dopamine receptor targets or diminished release of dopamine by dopaminergic amacrine cells. In support of this latter possibility, studies in rats and mice have found that the total retinal dopamine content is significantly decreased after several weeks of induced diabetes (Aung et al., 2014; Nishimura and Kuriyama, 1985), although findings by Lahouaoui et al. (2016) suggest that this difference may depend on circadian time point. Additionally, multiple studies performed in rodent models of early diabetes have identified a decline in dopaminergic amacrine cell number (Gastinger et al., 2006; Seki et al., 2004; Szabadfi et al., 2012), as early as 3 weeks in rats (Szabadfi et al. 2012) and as early as 3 months in mice (Lahouaoui et al., 2016). However, it is still unclear how this early dopamine deficiency affects dopaminergic signaling, and whether death of dopaminergic amacrine cells is a significant aspect of early diabetes.

Thus, the goal of this study was to determine how light adaptation and dopamine sensitivity are affected in early diabetes. We examined these phenomena in the context of the rod pathway, as current evidence points to its disruption in early diabetes (Aung et al., 2014; Castilho et al., 2015a; Moore-Dotson et al., 2016; Moore-Dotson and Eggers, 2019; Pardue et al., 2014). Previous work from our lab has shown that rod bipolar cells in diabetic mice receive less evoked inhibition than those in control animals (Moore-Dotson et al., 2016;

Moore-Dotson and Eggers, 2019), which could explain the hyper-excitability of ON ganglion cells that has been reported in an animal model of early diabetes (Cui et al., 2019; Yu et al., 2013). In the wild-type retina, this inhibition is controlled by the activation of dopamine type-1 receptors (D1Rs) located on amacrine cells (Flood et al., 2018). Thus, this study focused on potential changes in dopamine and light adaptation of rod bipolar cell inhibition, as well as the effects of these changes on excitatory input at the ganglion cell level. It was also determined if these changes in retinal activity could be explained by loss of specific retinal neuron types in diabetes.

2. Material and Methods

2.1 Animals

Animal protocols conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Visual Research and were approved by the University of Arizona Institutional Animal Care and Use Committee. Experiments used C57BL/6J male mice (Jackson Laboratories, Bar Harbor, ME, USA) that were housed in the University of Arizona animal facility and given the National Institutes of Health-31 rodent diet food and water ad libitum. Five-week-old mice were fasted for 4 hours and injected intraperitoneally with either streptozotocin (STZ, Sigma-Aldrich Corp., St. Louis, MO, USA; 75 mg/kg body weight) dissolved in 0.01 M pH 4.5 citrate buffer or citrate buffer vehicle for three consecutive days. Body weight and urine glucose were monitored weekly. Six weeks after the first injection, mice were fasted for 4 hours and blood glucose was measured (OneTouch UltraMini; LifeScan, Milpitas, CA, USA). STZ-injected animals with blood glucose ≥ 200 mg/dL and control animals with blood glucose ≥ 200 mg/dL were eliminated from the study. Fasting blood glucose was 154 ± 4 mg/dL ($n=38$ mice) for control mice and 414 ± 13 mg/dL ($n = 43$ mice; $P < 0.001$) for STZ-treated mice. STZ treated mice used in this study are henceforth referred to as diabetic. Body weights of control and diabetic mice were 25.1 ± 0.4 and 20.5 ± 0.3 g ($p < 0.001$), respectively.

2.2 Retinal tissue preparation

As previously described (Eggers and Lukasiewicz, 2006), mice were dark adapted overnight and both eyes were enucleated in the dark. For each mouse, the right eye was used for electrophysiology and the left was used for immunostaining. For electrophysiology, the cornea and lens were removed and the eyecup was incubated in cold extracellular solution (see Solutions and drugs) with 800 units/mL of hyaluronidase (Sigma; St. Louis MO) for 20 minutes to remove residual vitreous. The eyecup was washed with cold extracellular solution and the retina was removed. The retina was trimmed into an approximate rectangle and mounted onto a $0.45 \mu\text{m}$ nitrocellulose round filter paper (Millipore Billerica, MA, USA). The filter paper containing the retina was transferred to a hand chopper and cut into $250 \mu\text{m}$ thick slices. Each slice was rotated 90° and mounted onto a glass cover slip using vacuum grease. All dissections and light response recording procedures were performed under infrared illumination to preserve the light sensitivity of our preparations. For immunostaining, the dissection was done under white light in extracellular solution bubbled with $95\% \text{O}_2/5\% \text{CO}_2$. Four radial cuts were made to enable the retina to lay flat on filter paper prior to fixation in 4% paraformaldehyde (diluted from 16%, Electron Microscopy

Solutions #15710) in PBS pH 7.4 for 30 minutes. For tyrosine hydroxylase (TH) staining, retinas were fixed in the eyecup before extraction to minimize time between enucleation and fixation. For the activated caspase-3 staining positive control, retina was extracted from a GFP-negative mouse from a mouse line that expresses GFP under the gustducin promoter (Huang et al., 2003; Lin and Masland, 2005), incubated in extracellular solution containing staurosporine (2 μ M), a non-specific kinase inhibitor known to induce apoptosis through caspase-3, for 4 hours in an oxygenated dark box and rinsed in fresh extracellular solution for 30 minutes. It was then fixed and stained.

2.3 Electrophysiology solutions, drugs, and recordings

Extracellular solution used as a control bath for dissection and whole cell recordings was bubbled with a mixture of 95%/5% O₂/CO₂ and contained (in mM): 125 NaCl, 2.5 KCl, 1 MgCl₂, 1.25 NaH₂PO₄, 20 Glucose, 26 NaHCO₃, 2 CaCl₂. The intracellular solution in the recording pipette used for monitoring inhibitory and excitatory currents contained (in mM): 120 CsOH, 120 Gluconic Acid, 1 MgCl₂, 10 HEPES, 10 EGTA, 10 TEA-Cl, 10 phosphocreatine-Na₂, 4 Mg-ATP, 0.5 Na-GTP, 50 μ M Alexa Fluor 488 (Invitrogen, Carlsbad, California, USA) and was adjusted to pH 7.2 with CsOH. With these concentrations, the reversal potentials for Cl⁻ and cation currents were calculated as -60 and 0 mV, respectively. The D1R agonist SKF-38393 (SKF, 20 μ M, Tocris, Bristol, United Kingdom) was used to activate D1 receptors selectively to mimic the effects of light adaptation. SKF was diluted in extracellular solution to the given concentration and applied to the bath during the recordings by a gravity-driven superfusion system (Cell Microcontrols, Norfolk, VA) at a rate of ~1mL/minute. All chemicals were purchased from Sigma-Aldrich (St. Louis, Missouri), unless otherwise indicated.

All light response recordings began with retinal slices in a dark-adapted state, followed by recordings performed under drug-added or light-adapted conditions (see Light Stimuli). Retinal slices on glass cover slips were placed in a custom chamber and heated to 32° by temperature controlled thin stage and inline heaters (Cell Microcontrols, Norfolk, VA). For SKF experiments, dark-adapted light intensity responses were measured first, followed by a 5 minute incubation period with SKF, after which light responses were again measured in the presence of SKF. For light adaptation experiments, dark-adapted light response measurements were followed by a 5 minute exposure to a rod-saturating background light, before again recording light responses (while maintaining the applied background light.) Whole-cell voltage clamp recordings from rod bipolar cells and ganglion cells in retinal slices were made as previously described (Eggers and Lukasiewicz, 2006). Light-evoked and spontaneous inhibitory postsynaptic currents (IPSCs) were recorded from rod bipolar cells clamped at 0 mV, to isolate inhibitory chloride currents. Light-evoked excitatory postsynaptic currents (EPSCs) were recorded from ON-s ganglion cells clamped at -60 mV, to isolate excitatory cation currents. Electrodes were pulled from borosilicate glass (World Precision Instruments, Sarasota, Florida, USA) using a P97 Flaming/Brown puller (Sutter Instruments, Novato, California, USA) and had resistances of 5-7 M Ω for rod bipolar cell experiments and 3-5 M Ω for ganglion cell experiments. Liquid junction potentials of 20 mV, calculated with Clampex software (Molecular Devices, Sunnyvale, California, US), were corrected for prior to recording. Light responses and spontaneous currents were sampled at

10kHz and filtered at 6 kHz with the four-pole Bessel filter on a Multi-clamp 700B patch-clamp amplifier (Molecular Devices, Sunnyvale, California, USA) and digitized with a Digidata 1140 data acquisition system (Molecular Devices, Sunnyvale, California, USA) and Clampex software.

2.4 Light stimuli

Full-field light stimuli were evoked with a light-emitting diode (LED; Agilent HLMP-3950, $\lambda_{\text{peak}} = 525$ nm, Palo Alto, CA) that was calibrated with an S471 optometer (Gamma Scientific, San Diego, CA, USA) and projected through the camera port of the microscope. The stimulus intensities were chosen to activate primarily rods (rod-dominant intensities, 9.5, 95, 950 photons $\cdot\mu\text{m}^{-2}\cdot\text{s}^{-1}$) and to activate primarily cones (cone-dominant intensities, $9.5\cdot 10^3$, $9.5\cdot 10^4$ and $9.5\cdot 10^5$ photons $\cdot\mu\text{m}^{-2}\cdot\text{s}^{-1}$). These intensities were calculated to be equivalent to 4.75, 47.5, 475, $4.75\cdot 10^3$, $4.75\cdot 10^4$, and $4.75\cdot 10^5$ R*/rod/s, respectively (Field and Rieke, 2002). Sequential light responses were recorded with a stimulating interval of 30 s. Stimulus intensities, background rod-saturating light (950 photons $\cdot\mu\text{m}^{-2}\cdot\text{s}^{-1}$), and duration (30ms) were controlled with Clampex software by varying the current through the LED. The background intensity was chosen as it was shown to maximally activate rods (Wang and Kefalov, 2009).

2.5 Cell classification

During whole cell recordings, cells were passively filled with Alexa Fluor 488 dye included in the intracellular solution. Confirmation of rod bipolar cell morphology (Ghosh et al., 2004) or ganglion cell ON sublamina processes termination was done at the end of each recording using an Intensilight fluorescence lamp and Digitalsight camera controlled by Elements software (Nikon Instruments, Tokyo, Japan). Ganglion cells were further characterized by their light-evoked EPSC resulting from a 500 ms duration $9.5\cdot 10^5$ photons $\cdot\mu\text{m}^{-2}\cdot\text{s}^{-1}$ flash of light. They were classified as ON-sustained if a light-evoked EPSC coincided with the onset of light, did not return to baseline until after light offset, and did not possess a distinct OFF response as well.

2.6 Electrophysiology Data Analysis

Two to four traces of light-evoked responses for each condition were low-pass Gaussian filtered (1000 Hz) and subsequently averaged using Clampfit (Molecular Devices, Sunnyvale, California, USA). The peak amplitude, charge transfer (Q), time to peak and decay to 37% of the peak (D37) were also determined using Clampfit. To ensure that any spontaneous events superimposed on evoked responses did not artificially inflate our peak amplitude calculations, we measured peaks as the maximum current values that did not overlap with a discernible spontaneous event. The bounds for integration used to calculate charge transfer were marked by the times at which the maximum response recorded for a cell began and when it returned to baseline, typically 1-2 seconds. The same integration bounds were used for all other responses in the same cell. The time to peak was calculated as the temporal difference between stimulus onset and the response peak amplitude. Since the decay time could not be easily fitted with a single or double exponential curve, the D37 was measured as the time it took for the IPSC or EPSC to decline from its peak amplitude to 37% of its peak amplitude.

For light-evoked IPSCs and EPSCs, data from each cell was normalized to the maximum response recorded from that cell under dark-adapted conditions. If there was no discernable response for a given light intensity after filtering and averaging, the peak amplitude was recorded as 0 and it was excluded from our analysis of response kinetics. Comparisons between experimental conditions and luminance intensities were made with two-way analysis of variance (ANOVA) tests using the Student-Newman-Keuls (SNK) method for pairwise comparisons in SigmaPlot (Systat software, San Jose, California, USA). If any data was shown to have a nonnormal distribution or unequal variance, tests were repeated on the $\log_{10}$ or square root (for peak amplitudes) values of data. Additionally, for each ganglion cell a transient/sustained response ratio was calculated from their excitatory response to a 500 ms duration flash of light by quantifying the charge transfer occurring within the first 100 ms after light onset and normalizing this to the total amount of charge transfer between light onset and the return of the light-evoked EPSC to baseline.

For spontaneous currents, events were included in the analysis if they occurred in the time between the light response returning to baseline and one second before the next light stimulus. Frequency, amplitude, inter-event interval (IEI) and 37% decay time constants (τ) were calculated using MiniAnalysis software (Synaptosoft, Fort Lee, NJ, USA). All events were manually selected by the same individual with the detection threshold set to 3.5 pA. If any event occurred before a previous event had returned to baseline, both were included in calculations of frequency and inter-event intervals but were excluded from amplitude and decay tau analysis. Decay τ 's were fit with a single exponent. Effects of treatments on spontaneous IPSCs were analyzed at the single cell level with Kolmogorov-Smirnov (KS) tests in Clampfit. Amplitude, inter-event interval, and τ histogram distributions were normalized to the number of events. Effects between groups were analyzed with paired or unpaired t-tests after normalizing each cell to its dark-adapted state. Individual cells were only included in the analysis if they had 10 or more spontaneous events per treatment condition. For all tests, differences were considered significant when $p \leq 0.05$. All data are reported as mean $\pm$ standard error of the mean (SEM).

2.7 Immunohistochemistry

After fixation, retinas were removed from filter paper, washed in PBS, and incubated overnight at 4°C under gentle agitation in blocking solution. For tyrosine hydroxylase/cleaved caspase-3 co-staining, the blocking solution contained 5% normal goat serum and 0.5% Triton X-100 in PBS. For ganglion cell/amacrine cell staining, the blocking solution contained 10% rabbit serum (Novus Biologicals) 5% donkey serum (Jackson ImmunoResearch), 0.5% Triton X-100 and 0.1% Bovine Serum Albumin in PBS. Retinas were incubated with primary antibodies diluted in blocking solution for 5 days under gentle perturbation at 4°C (Table 1). After three one-hour washes in PBS, retinas were incubated overnight at 4°C with appropriate secondary antibodies diluted in PBS: goat anti-mouse IgG2a AlexaFluor488 (1:1000, Thermo Fisher Scientific #A21131), donkey anti-rabbit AlexaFluor546 (1:1000, Thermo Fisher Scientific #A10040), rabbit anti-mouse IgG2a Dylight 488 (1:500, Novus Biologicals #NBP1-72891), rabbit anti-goat Dylight 550 (1:500, Novus Biologicals #NBP1-76149), and donkey anti-guinea pig Alexa Fluor 647 (1:4000, Jackson Immuno Research #706-605-148). The nuclear stains TO-PRO-3 Iodide (1:4000,

Invitrogen #T3605) or DAPI (1 µg/ml, Thermo Fisher Scientific #D1306 for anti-RBPMS immunostaining) were also included during secondary antibody incubation. Following the secondary antibody incubation, the tissue underwent three one-hour washes in PBS. Tissues were then mounted on microscope slides using Slowfade mounting medium (Thermo Fisher Scientific) and sealed with clear fingernail polish.

2.8 Imaging and Data Analysis

To look for changes in the density of TH-positive cells, images of whole mount retinas were acquired on a Zeiss LSM 880 inverted confocal microscope (Carl Zeiss Microscopy, Oberkochen, Germany) with a 10x objective (Plan-Apochromat 10x/0.45) 700 µm away from the optic nerve head using an 850.19 µm x 850.19 µm field of view. Maximum Z-projections were used to count numbers of TH-positive cells in the inner nuclear layer (INL) in all four quadrants of each retina using the ImageJ Cell Counter plug-in (<http://rsbweb.nih.gov/ij/plugins/cell-counter.html>; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA). Only the cells with nuclei more than 50% within the borders of the region of interest were counted. The cell numbers were averaged amongst the four locations in each retina, normalized for the area of the region of interest, and averaged with all the retinas of the corresponding group (i.e., diabetic or control).

To determine the average soma size, maximum Z-projections of images acquired 600 µm from the optic nerve head with a 20x objective (Plan-Apochromat 20x/0.8) were used to measure the area of each TH-positive cell in the INL. Image J's freehand tool and ROI Manager were used to circumscribe and measure each soma. The mean soma area in each quadrant was calculated and data was analyzed as above. Activated caspase-3 staining was analyzed from maximum Z-projections of the same image stacks as above, including ganglion cell layer (GCL), inner nuclear layer (INL) and outer plexiform layer (OPL). Caspase-positive cells were counted in each quadrant of each layer and data was analyzed as described above.

To determine the relative TH staining intensity of dopaminergic amacrine cells in the INL, whole mount images were acquired 500 µm away from the optic nerve head with a 40x objective (Plan-Apochromat 40x/1.3 oil). ImageJ was used to quantify mean TH intensity from background-subtracted single slices from these image stacks. Data was analyzed as above.

To determine the numbers of ganglion and amacrine cells in each layer, whole mount retinas were imaged with a 40x objective. Stacked retinal images-were acquired from four regions (temporal, nasal, dorsal, ventral) 600 µm from the optic nerve head; the region of interest of each captured image was 212.55 µm x 212.55 µm. Cells were counted using the Image J cell counter plug-in. A different marker was used to mark unique cells (of each cell type) in each vertical (Z-axis) layer to prevent double counting. Only the cells with nuclei more than 50% within the borders of the region of interest were counted. Data was analyzed as above.

A negative control for the imaging experiments was done to test the specificity of each of the primary antibodies using the same procedures, imaging, and analysis as the experimental eyes without primary antibody incubation. Experiments done without primary antibody

showed no labelling of the targeted cells of interest, demonstrating the specificity of our primary antibodies. For all imaging data, unpaired Student's T-tests were used to determine if there was a significant difference between treatment groups. Differences between the two groups were considered significant if $P < 0.05$. All results are reported as the mean $\pm$ SEM.

3. Results

3.1 Diabetes disrupts inhibitory rod bipolar cell and excitatory ON-sustained ganglion signaling

A previous study found that light-evoked inhibition to rod bipolar cells was significantly diminished after 6 weeks of diabetes (Moore-Dotson et al., 2016). To confirm these previous findings light-evoked IPSCs were measured from rod bipolar cells (see section 2.5) in control and diabetic retinas. Diabetes significantly reduced light-evoked IPSC peak amplitude (2-way ANOVA; control $n=15$ cells, diabetic $n=15$; $p=0.005$, Fig. 1A, B), but not charge transfer (2-way ANOVA; control $n=15$, diabetic $n=15$, $p=0.123$, Fig. 1C). Additionally there was no significant difference in time to peak or D37 (2-Way ANOVA; control $n=13$, diabetic $n=11$; Time to Peak $p=0.746$, D37 $p=0.282$, Fig 1D, E). This loss of inhibition could lead to increased bipolar cell inputs to ganglion cells.

To test this, dark-adapted light-evoked EPSCs in ON-sustained (ON-s, see section 2.5) ganglion cells were measured in control and diabetic animals (Fig. 2A). The peak amplitude of ganglion cell light-evoked EPSCs significantly increased (2-way ANOVA; control $n=14$, diabetic $n=18$; $p=0.013$, Fig. 2B). There were no significant differences in charge transfer, time to peak, or D37 (2-way ANOVA; control $n=14$, diabetic $n=18$; charge transfer $p=0.611$, time to peak $p=0.860$, D37 $p=0.870$, Fig. 2C–E). However, as these recordings were performed in retinal slices it is possible that any differences in absolute response magnitudes are due to variability in the number of inputs to the cells in each sample. Thus, dark-adapted responses were normalized to the largest response for each cell to determine how responses changed with increasing light intensity, independent of absolute response. Normalized diabetic light-evoked EPSCs were significantly different than control light-evoked EPSCs in both peak amplitude and charge transfer (2-way ANOVA, control $n=14$, diabetic $n=18$; peak amplitude $p=0.011$, charge transfer $p=0.005$, Fig. 2F, G). Interestingly, these differences arose from diabetic responses being larger in pairwise comparisons at 95 and 950 photons $\mu\text{m}^{-2}\cdot\text{s}^{-1}$ (SNK, peak amplitude: 95 $p=0.037$, 950 $p=0.001$; charge transfer: 95 $p=0.028$, 950 $p=0.001$), light intensities that activate the rod pathway to a greater degree than the cone pathway. There were no significant differences found between control and diabetic light-evoked EPSCs in normalized time to peak or D37 (2-way ANOVA; control $n=14$, diabetic $n=18$; time to peak $p=0.223$, D37 $p=0.074$, Fig. 2H, I). Additionally, to evaluate the similarity of the control and diabetic ganglion cell populations, the transient and sustained components of each cell in response to a longer 500 ms light stimulus (Fig. 2J–K) were measured. There was no significant difference between the transient/sustained ratios of control and diabetic cells (Fig. 2K). These results suggest a functionally relevant change to ganglion cell inputs in the dark-adapted retina after 6 weeks of diabetes, likely from altered rod pathway signaling.

3.2 D1R dependent modulation of rod pathway light-evoked inhibition is unaffected by 6 weeks of diabetes

In wild-type retinas, D1R activation causes significant reductions in peak amplitude and charge transfer of dark-adapted light-evoked IPSCs in rod bipolar cells (Flood et al., 2018). To determine if D1R function is compromised in diabetes by loss of dopamine, the effects of the partial D1R agonist SKF-38393 on inhibition to dark-adapted rod bipolar cells was measured (Fig. 3). Treatment with SKF significantly decreased the peak amplitude and charge transfer of both control and diabetic rod bipolar cell light-evoked IPSCs (2-way ANOVA; control $n=6$; peak amplitude $p<0.001$, charge transfer $p<0.001$, diabetic $n=9$ peak amplitude $p<0.001$, charge transfer $p<0.001$; Fig. 3B, C). SKF treatment did not cause any significant changes in time to peak or D37 values in either control or diabetic rod bipolar cells (2-way ANOVA; control $n=4$; time to peak $p=0.066$, D37 $p=0.164$, diabetic $n=6$; time to peak $p=0.056$, D37 $p=0.076$; Fig. 3D, E). These data suggest that cells in both control and diabetic retinas are still responding to a D1R dopamine agonist. However, there was no significant effect of disease state on normalized SKF responses (peak amplitude $p=0.092$, charge transfer $p=0.135$, time to peak $p=0.873$, D37 $p=0.625$, Fig. 3B). Overall, these results suggest that dopamine sensitivity and responsivity at the rod bipolar cell level is not compromised at this stage of early diabetes.

3.3 D1R modulation of spontaneous rod bipolar cell inhibition is largely intact after 6 weeks of diabetes

Spontaneous inhibitory currents in rod bipolar cells are relatively low frequency, but dopamine modulation of basal rod bipolar cell inhibition may be important for signaling (Herrmann et al., 2011; Smith et al., 2015). A previous study showed that SKF treatment decreases spontaneous IPSCs in wild-type rod bipolar cells, reflecting diminished release by amacrine cells onto rod bipolar cells upon D1R activation (Flood et al., 2018). To determine if diabetes changes this D1R modulation, spontaneous IPSCs were measured from dark-adapted control and diabetic rod bipolar cells before (Fig. 4A, Table 2) and after SKF treatment (Fig. 4B). SKF significantly decreased average spontaneous IPSC frequency for both control ($n=6$, $p<0.001$, Fig. 4B1) and diabetic rod bipolar cells ($n=6$, $p=0.027$) and significantly increased inter-event intervals for 4/6 control cells and 5/6 diabetic cells (K-S, $p<0.05$, Fig. 4C). There was no significant change in average spontaneous IPSC amplitude for either group (control $p=0.600$, diabetic $p=0.924$, Fig. 4B2, D). There was also no significant change in average spontaneous IPSC decay τ (control $p=0.116$, diabetic $p=0.471$, Fig. 4B3, E). However, similar to the rod bipolar cell light-evoked IPSCs, there were no significant differences between the average effects of SKF between the control and diabetic groups (unpaired t-tests, $p>0.05$, Fig. 4B). These results suggest that diabetes is not significantly affecting the capability of dopamine to modulate basal inhibition to rod bipolar cells.

3.4 No significant impairment in light adaptation of inhibition is detected at the rod bipolar cell level after 6 weeks of diabetes

Although there was no significant impairment in the ability of the retina to respond to a D1R agonist, it is possible that the capacity of diabetic retinas to release and respond to

endogenous dopamine is compromised. Light-evoked IPSCs were measured before and after exposing retinal slices to 5 minutes of a light-adapting background illumination, which leads to retinal dopamine release (Witkovsky, 2004). In wild-type retinas, this treatment results in significant reductions to light-evoked IPSCs (Eggers et al., 2013). Here, light adaptation had similar effects on light-evoked IPSCs in both control and diabetic rod bipolar cells (Fig. 5A), causing significant reductions in peak amplitude and charge transfer (2-way ANOVA; control $n=7$, peak amplitude $p<0.001$, charge transfer $p<0.001$; diabetic $n=6$; peak amplitude $p<0.001$, charge transfer $p<0.001$, Fig. 5B, C). Light adaptation did not significantly affect time to peak (2-way ANOVA; control $n=4$, $p=0.108$, diabetic $n=3$, $p=0.513$; Fig. 5D) but did significantly decrease D37 values (2-way ANOVA; control $n=4$, $p=0.036$, diabetic $n=3$, $p<0.001$; Fig. 5E). However, there were no significant differences in the effect of light adaptation on peak amplitude (2-way ANOVA; $p=0.944$), charge transfer (2-way ANOVA, $p=0.147$), time to peak (2-way ANOVA, $p=0.874$), or D37 (2-way ANOVA, $p=0.086$), between control and diabetic rod bipolar cells.

In wild-type retinas, light adaptation also causes a significant reduction in spontaneous IPSCs (Eggers et al., 2013). To determine if this is impaired in diabetic retinas, the light-adapted changes in spontaneous IPSC frequency, amplitude, and decay τ were quantified in control and diabetic rod bipolar cells (Fig. 6B). Light adaptation significantly decreased the average frequency of spontaneous IPSCs in both control and diabetic rod bipolar cells (paired t-test; control: $n=5$, $p=0.0003$; diabetic: $n=5$, $p=0.016$, Fig. 6B1, Table 3). At the single cell level 5/5 control and 3/4 diabetic rod bipolar cells exhibited significant shifts in their spontaneous IPSC inter-event intervals towards longer values (Fig. 6C). Although there was no significant change in average spontaneous IPSC amplitude after light adaptation (paired t-test; control: $n=5$, $p=0.109$; diabetic: $n=4$, $p=0.816$, Fig. 6B2), 4/5 control and 2/4 diabetic rod bipolar cells exhibited spontaneous IPSC shifts towards smaller amplitudes (K-S, $p<0.001$, Fig. 6D). There was also no change in average spontaneous IPSC decay τ after light adaptation (paired t-test; control: $n=5$, $p=0.904$; diabetic: $n=4$, $p=0.713$, Fig. 6B3, E). Similar to light-evoked IPSCs, there were no significant differences in the relative changes due to light adaptation between control and diabetic spontaneous IPSCs (Fig. 6B, unpaired t-test; frequency $p=0.887$, amplitude $p=0.236$, decay τ $p=0.738$). Altogether, the data on SKF and light adaptation of rod bipolar cell light-evoked and spontaneous IPSCs suggests that at the rod bipolar cell level dopaminergic signaling is mostly normal in the diabetic retina, despite lowered retinal dopamine levels.

3.5 Modulation of ganglion cell excitatory inputs by light adaptation is impaired after 6 weeks of diabetes

Although there were no significant defects in dopaminergic signaling and light adaptation in diabetic retinas at the level of the rod bipolar cell, it is possible that dysfunction could happen in other areas of the retinal circuit. Ganglion cells are the targets of a large degree of convergence between bipolar cell outputs, so measurements made in these cells would be most sensitive to any potential perturbation in dopaminergic signaling. To test this, light-evoked EPSCs in ON-s ganglion cells were measured before and after light adaptation in control and diabetic animals (Fig. 7A). Light adaptation caused significant reductions in light-evoked EPSC peak amplitude and charge transfer (2-way ANOVAs; control $n=10$, peak

amplitude $p < 0.001$, charge transfer $p < 0.001$, diabetic $n = 11$, peak amplitude $p < 0.001$, charge transfer $p < 0.001$; Fig. 7B, C). Light adaptation did not cause significant changes in time to peak (2-way ANOVA; control $n = 8$, $p = 0.095$, diabetic $n = 10$, $p = 0.072$; Fig. 7D), but did significantly decrease D37 (2-way ANOVA; control $n = 8$, $p < 0.001$, diabetic $n = 10$, $p = 0.006$; Fig. 7E). Interestingly, light-adapted reductions in diabetic ON-s ganglion cell charge transfer were significantly smaller than in control ($P = 0.014$), but there was no significant difference in the effect of light adaptation on peak amplitude ($p = 0.664$), time to peak ($p = 0.333$) or D37 ($p = 0.497$). Therefore, at the ganglion cell level the lack of dopamine in diabetes causes weakened light adaptation.

To determine whether these changes in light-evoked EPSCs could be attributed to pre- or postsynaptic modifications, spontaneous EPSCs were also analyzed in control and diabetic ganglion cells before and after light adaptation (Fig. 8A). Light adaptation caused a significant decline in spontaneous EPSC frequency for both control (paired t-test, $n = 10$, $p = 0.0283$) and diabetic (paired t-test, $n = 10$, $p = 0.0008$) ganglion cells (Fig. 8B1, Table 4). This was due to significant shifts in spontaneous EPSC inter-event intervals towards longer values in 7/10 control and 8/9 diabetic ganglion cells (Fig. 8C). Light adaptation did not cause a significant decline in mean spontaneous EPSC amplitude for either control or diabetic ganglion cells (paired t-test, control $p = 0.991$; diabetic $p = 0.295$, $n = 10$ for both groups, Fig. 8B2, D). There was also no significant change in mean decay τ due to light adaptation (paired t-test, control $p = 0.533$; diabetic $p = 0.603$, $n = 10$ for both control and diabetic groups, Fig. 8B3, E). These results suggest that the main site of light adaptation of ganglion cell EPSCs is upstream of the ganglion cell. However, unlike the light-evoked EPSCs there were no significant differences in the mean normalized changes after light adaptation in spontaneous EPSC frequency (unpaired t-test, $p = 0.314$), peak amplitude (unpaired t-test, $p = 0.470$), or decay τ (unpaired t-test, $p = 0.973$) between control and diabetic animals (Fig. 8B), suggesting no effect of disease state on light adaptation of spontaneous EPSCs.

3.6 Dopaminergic amacrine cell numbers are maintained after 6 weeks of diabetes

This loss of light adaptation capability in the diabetic ganglion cells and the reported loss of dopamine in the diabetic mouse retina could be due to loss of dopaminergic amacrine cells in the retina that release dopamine. To determine whether dopaminergic amacrine cells are lost, the number of cells positive for tyrosine hydroxylase (TH) and apoptosis marker activated caspase-3 were quantified. There was no difference in the density of dopaminergic (TH+) amacrine cells between diabetic and control eyes (unpaired t-test, control $n = 7$ retinas, diabetic $n = 6$ retinas; $p = 0.535$, Fig. 9A1, B1, Table 5). There were also no differences in soma size (unpaired t-test, control $n = 4$, diabetic $n = 4$; $p = 0.124$, Fig. 9B2) or soma TH signal intensity (unpaired t-test, control $n = 3$, diabetic $n = 3$; $p = 0.994$, Fig. 9B3). Additionally, there was no evidence for apoptosis occurring in the inner nuclear layer in either control or diabetic retinas. A small number of caspase-positive cells were found in the ganglion cell layer (GCL) and outer plexiform layer (OPL) of both control and diabetic retinas, although the numbers for each layer are not significantly different between treatment groups (data not shown). These results suggest that impaired dopamine levels are not due to loss of dopaminergic amacrine cells.

3.7 Diabetic retinas show no significant cell loss of ganglion or amacrine cells after six weeks of diabetes

It is possible that changes in ganglion cell signaling could be due to loss of ganglion cells. To determine this, retinas were stained with either DAPI or TO-PRO-3 cell nuclei stains to label the total cell population in the GCL and antibodies against RBPMS and GAD 65/GAD 67 to specifically label the ganglion cells and GABAergic displaced amacrine cells (Jeon et al., 1998) found in the GCL (Fig. 10A–B). An antibody against GlyT1 was also used to label glycinergic amacrine cells, but no staining was observed in the GCL (data not shown). There was no overlap in RBPMS and GAD65/67 staining, so neurons were counted in the same images and labeled as either a ganglion cell stained with RBPMS or a displaced GABAergic amacrine cell. There was also a small population of cells in the GCL that were not labeled with either RBPMS or GAD antibodies ($12.9 \pm 0.9\%$ control retinas; $9.0 \pm 1.5\%$ diabetic retinas) that are presumed to be ganglion cells. There was no significant difference between control and diabetic retinas in RBPMS-labeled ganglion cell density (Table 6, Fig. 10.C1), displaced amacrine cell density (Fig. 10.C2), or total GCL cell density (Fig. 10.C3). These results show there is no loss in total neurons in the ganglion cell layer or in ganglion cell or displaced amacrine cell subpopulations after 6 weeks of diabetes.

It is also possible that loss of rod bipolar cell inhibition could be due to loss of inhibitory amacrine cells. GAD65/67 and GlyT1 antibodies labeled GABAergic and glycinergic amacrine cells, respectively, in the inner nuclear layer (INL, Fig. 10D–E). Amacrine cells were either labeled by GAD65/67 or GlyT1, as shown previously (Haverkamp and Wässle, 2000), and were quantified together to ensure an accurate accounting of all amacrine cells. No difference between control and diabetic retinas was found in GABAergic (Fig. 10F1), glycinergic (Fig. 10F2), or total (Fig. 10F3) amacrine cell densities in the INL. These results show that loss of inhibition to rod bipolar cells after 6 weeks of diabetes is not due to loss of amacrine cells.

4. Discussion

These results show two main important findings. First, after 6 weeks of diabetes, ganglion cell function is significantly compromised, particularly at rod-dominant light levels. This agrees with our results here confirming dysfunctional rod bipolar cell inhibition and other studies suggesting impaired rod pathway signaling in diabetes (Aung et al., 2014; Castilho et al., 2015a; Moore-Dotson et al., 2016; Moore-Dotson and Eggers, 2019; Pardue et al., 2014). Second, we showed that light adaptation capacity in ganglion cells is reduced, which could be due to reduced dopamine release due to previously reported reductions in dopamine levels in the diabetic retina (Aung et al., 2014; Nishimura and Kuriyama, 1985). All of these changes happened without any loss of ganglion cells, dopaminergic, GABAergic or glycinergic amacrine cells. This suggests that a disruption to neuronal signaling and modulation is responsible for the earliest detectable signs of retinal dysfunction in diabetes.

4.1 ON-s ganglion cells receive increased peak excitation under dark-adapted conditions after 6 weeks of diabetes

Many diabetic patients who exhibit no clinical signs of vascular retinopathies have impaired contrast sensitivity (Di Leo et al., 1992; Dosso et al., 1996; Ewing et al., 1998; Hyvarinen et al., 1983), especially under dim-light conditions (Katz et al., 2010; Lopes de Faria et al., 2001; Safi et al., 2017), which could suggest changes to retinal ganglion cell function. While previous studies (Castilho et al., 2015a; Moore-Dotson et al., 2016) showed that disrupted inhibition at rod bipolar cell terminals increased excitatory currents to AII amacrine cells, it is not known if this leads to changes at the level of retinal ganglion cells. Here we showed that ON-s ganglion cells, which receive the majority of their excitatory input from ON-6 bipolar cells (Schwartz et al., 2012; Tien et al., 2017) that are highly coupled to AII amacrine cells (Tsukamoto and Omi, 2017), receive increased peak excitation in response to light stimuli in 6-week diabetic mice (Fig. 2B). This demonstrates that at least one channel of retinal output to the lateral geniculate nucleus may be dysfunctional at this early stage of diabetes. This finding is supported by previous work reporting hyper-excitability of background activity of dark-adapted ON ganglion cell after 12 weeks of diabetes, due to increased spontaneous spiking, changes in passive membrane properties and voltage-gated conductances (Cui et al., 2019; Yu et al., 2013).

The correlation of increased inputs to ganglion cells with decreased inhibition to rod bipolar cells that is shown here and in previous studies (Castilho et al., 2015a; Moore-Dotson et al., 2016; Moore-Dotson and Eggers, 2019), suggests that decreased inhibition may underlie the increased ganglion cell responses. However, there are other potential explanations for increased ganglion cell inputs. Previously, dendritic expansion for some populations of ON ganglion cells has been reported in Ins Akita mice after 12 weeks of diabetes (Gastinger et al., 2008), a change that could result in more excitatory inputs and stronger responses to stimuli. However, in contrast to our study (Fig. 10, Table 6) the ganglion cell changes were accompanied by significant ganglion cell death (16.4% of those under consideration) that could trigger expansion of the remaining ganglion cell dendrites. Additionally, a different study showed a decreased in dendritic area in a subtype of ON ganglion cells without cell loss (Cui et al., 2019) after 12 weeks of diabetes, suggesting dendritic expansion is unlikely in our sample. Alternatively, there is some evidence for early diabetic changes in glutamate receptor subunit composition and glutamate transporter expression in the retina (Castilho et al., 2015a; Castilho et al., 2015b; Lau et al., 2013; Santiago et al., 2009; Santiago et al., 2008) that could potentially lead to increased inputs to ganglion cells. In support of these possibilities, we found dark-adapted diabetic spontaneous EPSCs had longer average decay τ 's compared to controls (2.63 ± 0.40 ms vs. 1.49 ± 0.16 ms, data not shown) that could suggest a change in glutamate channel properties. However, we found no significant difference in light response kinetics (Fig. 2D–E) that would reflect a functional change attributable to these potential single channel/transporter modifications.

4.2 Light adaptation and dopaminergic modulation of rod bipolar cell inhibition remain unimpaired in early diabetes

The amount of inhibition rod bipolar cells receive can be modulated by changes in inhibition between amacrine cells. In the wild-type retina, light adaptation causes a significant

reduction in light-evoked inhibition to rod bipolar cells, likely in part by modulating the activity of these amacrine cell-inhibiting amacrine cells (Eggers et al., 2013). Recent studies (Flood et al., 2018; Smith et al., 2015; Travis et al., 2018) have demonstrated a role for D1 receptor activation in modulating the strength of lateral inhibition onto rod bipolar cells, possibly by potentiating GABA_A currents onto the amacrine cells responsible for lateral inhibition (Feigenspan and Bormann, 1994). Thus, it follows that a deficit in dopaminergic signaling could result in impaired light adaptation of lateral inhibition to rod bipolar cells.

In this study, we mimicked dopamine release onto D1 receptors by applying the partial D1 agonist SKF-38393, and saw significant reductions to rod bipolar cell light-evoked IPSCs in both control and diabetic animals, as expected by results from wild-type rod bipolar cells (Flood et al., 2018). However, we found no difference in the proportion of response reduction between the two groups (Fig. 3) and no major differences in the changes to spontaneous IPSCs between diabetic and control groups (Fig. 4), implying similar modifications to amacrine cell release upon D1 receptor activation. This suggested the sufficient expression of D1 receptors at the appropriate retinal sites to mediate this aspect of dopaminergic signaling after 6 weeks of diabetes. Changes in the relative expression of D1 receptors in other areas of the central nervous system have been identified during early stages of diabetes (Salkovic and Lackovic, 1992; Serri et al., 1985). In striatal rodent neurons D1 receptors are sequestered or show membrane accumulation in response to chronic hyper- or hypodopaminergic conditions, respectively (Dumartin et al., 2000; Martin-Negrier et al., 2000). Based on evidence for diminished dopamine levels in diabetic retinas (Aung et al., 2014; Kim et al., 2018; Nishimura and Kuriyama, 1985), we would expect D1 receptors to be localized to retinal neuronal membranes at normal or even supranormal levels if regulated in a similar fashion to those in other neural regions. This agrees with our results showing preserved D1R function.

However, it was surprising that light adaption affected diabetic and control rod bipolar cell light-evoked IPSCs (Fig. 5) or spontaneous IPSCs (Fig. 6) equally. Based on diminished dopamine levels in early diabetes (Aung et al., 2014; Kim et al., 2018; Nishimura and Kuriyama, 1985), diminished dopamine release in diabetic retinas in response to light stimulation (Nishimura and Kuriyama, 1985) and deficits in light adapted ERGs and visual function in mice that lack retinal dopamine (Jackson et al., 2012), we had hypothesized that dopamine release and thus light adaptation would be impaired in our diabetic animals. However, our results showed no deficit in light adaptation of rod bipolar cell light-evoked IPSCs. This suggests that dopamine release onto amacrine cells presynaptic to rod bipolar cells is either not reduced or still sufficient to provide adequate modulation at the rod bipolar cell level.

In agreement with these results, we also found no evidence for death or reduced TH function in dopaminergic amacrine cells (Fig. 9). Preserved dopaminergic amacrine cell number after 6 weeks of diabetes agrees well with the literature, as the earliest time point at which significant apoptosis has been reported in STZ-induced diabetic mice is 12 weeks (Lahouaoui et al., 2016). Although our results show that tyrosine-hydroxylase expressing amacrine cells are preserved, many other factors could still be affecting dopamine production and release. It would be helpful for future studies to measure the release of

dopamine from dopaminergic amacrine cells, to assess the time point at which function is negatively impacted.

4.3 Light adaptation of ON sustained ganglion cells is impaired in early diabetes

Light adaptation causes a reduction in ganglion cell excitability, shifting their intensity response curves to encode signals properly under increased ambient light conditions (Enroth-Cugell and Shapley, 1973; Ke et al., 2014). However, we showed that light adaptation of light-evoked excitatory inputs to diabetic ganglion cells was impaired (Fig. 7). This suggests a disruption in light adapting post-synaptic changes in ganglion cells and/or presynaptic ON bipolar cell release of glutamate. Dopamine, D1 and D2 receptor agonists can modulate ion currents in ganglion cells (Chen and Yang, 2007; Hayashida et al., 2009; Jensen, 2015; Ogata et al., 2012). Thus, the change we observed in ganglion cell signaling could be explained by impaired dopaminergic signaling to ganglion cells. However, it is difficult to tease apart the effects of dopamine at the ganglion cell level from its effects upstream. Dopamine receptors are expressed by photoreceptors, horizontal cells, some cone bipolar cells (including ON type-6 bipolar cells) and amacrine cells (Cohen et al., 1992; Derouiche and Asan, 1999; Farshi et al., 2016; Li et al., 2013; Veruki, 1997; Veruki and Wässle, 1996), and perturbed function at any or all of these sites could propagate down to the ganglion cell level. The most consistent finding from our analysis of spontaneous EPSCs was a reduction in spontaneous EPSC frequency following light adaptation (Fig. 8B1), suggesting a pre-synaptic mechanism as being responsible for light-induced changes in ganglion cell excitation.

If the excessive excitation after light adaptation is due to pre-synaptic changes, it could result from cone bipolar cells receiving too much excitation or insufficient inhibition, or from impaired dopaminergic action upon cone bipolar cells themselves. Because we saw no difference in the degree of spontaneous EPSC frequency reduction between control and diabetic cells after light adaptation, it seems likely that changes to ON bipolar cell inputs, and not changes in the direct effect of dopamine upon ON bipolar cells themselves, are the cause of this impaired light adaptation. In our mouse model of type 1 diabetes there are multiple reports of retinal dysfunction in dark-adapted ERG parameters occurring by 6 weeks of diabetes (Aung et al., 2014; Chang et al., 2019; Kim et al., 2018; Piano et al., 2016; Sergeys et al., 2019), suggesting dysfunction in the rod pathway. However, less is known about the ability of diabetic retinas to respond to brighter light levels. Two studies have reported no change in photopic ERG responses (Piano et al., 2016; Samuels et al., 2012) at 12 and 22 weeks following diabetes induction, while others have reported diminished amplitudes in photopic flicker responses after 5 and 12 weeks of diabetes (Aung et al., 2014; Ramsey et al., 2006). These results would imply that light-adapted excitatory input to cone bipolar cells is either unchanged or reduced early on in diabetes, which would not explain our current findings that inputs to diabetic ganglion cells were resistant to light adapted declines.

One potential explanation is that the rod pathway may be providing excessive excitation to ON bipolar cells after light adaptation. Recent studies have shown that the rod pathway remains at least somewhat active under the light-adapted conditions we employed in this

study (Pasquale et al., 2020; Tikidji-Hamburyan et al., 2017). Although diabetic rod bipolar cell light-evoked IPSCs were reduced similarly to control, they have smaller amplitudes in dark-adapted conditions, which would result in them remaining disinhibited under light-adapted conditions. Another possibility is that inhibitory signaling in the cone pathway is also affected at this early time point of diabetes. Significant delays in photopic oscillatory potentials have been recorded in STZ-induced diabetic mice after 5 weeks of diabetes (Kim et al., 2018), raising the potential for disrupted feedback to cone bipolar cell terminals. Additionally, as dopamine action via D1 receptors has been shown to modify the strength of cone bipolar cell surrounds (Chaffiol et al., 2017; Mazade and Eggers, 2016; Mazade et al., 2019), disrupted dopaminergic signaling at the level of inhibitory feedback to cone bipolar cells could also potentially explain our findings.

4.4 Changes in inhibitory and excitatory signaling after 6 weeks of diabetes are not attributable to cell death

For this study, we developed an immunohistochemical protocol for the simultaneous labeling of GABAergic amacrine cells, glycinergic amacrine cells, ganglion cells, and cell nuclei in the whole mount retina. This allowed us to differentiate between ganglion and displaced amacrine cells in the GCL and to take advantage of non-colocalization to identify the labeled cells with a high degree of confidence. Using this methodology, we have shown that at this early time point in diabetes there are no significant changes in the absolute or relative numbers of ganglion cells and displaced amacrine cells in the GCL, or GABAergic and glycinergic amacrine cells in the inner nuclear layer (INL, Fig. 10). There are differing reports in the literature regarding the timescale and degree of neuronal cell loss in early diabetes. While there is some evidence for significant ganglion cell death as early as 6-8 weeks after diabetes induction (Lobanovskaya et al., 2018; Yang et al., 2012), many studies suggest that significant ganglion cell death only occurs at later time points in the disease (Gastinger et al., 2008; Hombrebueno et al., 2014; Kern and Barber, 2008). After 12 weeks of diabetes, Yu et al. (2013) reported no loss of SMI-32 labeled retinal ganglion cells. Hombrebueno et al. (2014) additionally reported no significant death of glycinergic or GABAergic amacrine cells until 9 months after diabetes induction. Here, our findings suggest that neuronal cell death does not play a role in the decreased light-evoked inhibition from amacrine cells onto bipolar cells that we observed after 6 weeks of diabetes. It also implies that the increased excitatory inputs we recorded at the ganglion cell level are likely not attributable to dendritic expansion after ganglion cell death, since no ganglion cell loss was observed.

4.5 Conclusions

The data reported here suggest that inhibitory dysfunction in the rod pathway during early diabetes can result in significant changes to ON sustained ganglion cell responses under dim light conditions. Although we did not detect any irregularity in dopaminergic signaling or light adaptation in rod bipolar cells of diabetic animals, we did find that diabetic ON sustained ganglion cells had an impaired response to light adaptation. These changes could potentially explain some of the subtle visual deficits reported in early diabetic human populations, including contrast sensitivity (Dosso et al., 1996; Hyvarinen et al., 1983; Katz et al., 2010; Tsai et al., 2016) and night vision (Greenstein et al., 1993). Future work to

further characterize the mechanism(s) for these changes and to determine whether the enhanced excitability of these cells early in diabetes may play a causative role in the progression of diabetic retinopathy will be important to understand the pathology of early diabetic retinal damage.

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Highlights

- Early diabetes causes retinal deficits in neuronal signaling
- These deficits include decreased bipolar cell inhibition and dopamine release
- Diabetes deficits increase dark and light adapted retinal ganglion cell excitation
- Neuronal signaling changes are not a result of loss of neurons
- These changes could lead to overexcitation and damage of retinal ganglion cells

Figure 1.

Light-evoked inhibition to rod bipolar cells under dark-adapted conditions is reduced after 6 weeks of diabetes. A. Example traces of light-evoked IPSCs recorded at increasing light intensities (from left to right) in a control (A1) and diabetic (A2) rod bipolar cell. 30 ms light stimuli are represented as small gold bars below traces. B-C. Average peak amplitude (B) and charge transfer (Q, panel C) of light-evoked IPSCs from control (blue squares) and diabetic (red triangles) rod bipolar cells. D-E. Average time to peak (D) and 37% decay (E) values for light-evoked IPSCs from control (blue squares) and diabetic (red triangles) rod

bipolar cells. Control n=6 for B-C and n=5 for D-E. Diabetic n=9 for B-E. P values are reported for 2-way ANOVA main effect of diabetes, accompanied by an * if significantly different.

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Figure 2.

Light-evoked excitation to ON-s ganglion cells under dark-adapted conditions is increased after 6 weeks of diabetes. A. Example traces of light-evoked EPSCs recorded at increasing light intensities (from left to right) in a control (A1) and diabetic (A2) ON-s ganglion cell (gold bars are 30 ms light stimuli). B-E. Average peak amplitude (B), Q (C), time to peak (D) and D37 (E) of light-evoked EPSCs from control (blue squares) and diabetic (red triangles) ON-s ganglion cells. F-I. Same as in B-E, but with the data normalized to the maximum response. J1-2. Example traces of a 500 ms stimulus-evoked light-evoked EPSC

in a control (J1) and diabetic (J2) ganglion cell. The light and dark gray fill areas denote the transient and sustained components, respectively. K. Proportions of transient and sustained components making up the 500 ms stimulus light-evoked EPSCs in control and diabetic ganglion cells. The responses of all cells were predominantly sustained, and were not significantly different between the two groups. Control n=14 and diabetic n=19. P values are reported for 2-way ANOVA main effect of diabetes, and accompanied by an * if significant. † = significantly different pairwise comparison at a specific light intensity (SNK post-hoc).

Figure 3.

D1R signaling is not significantly impaired at the rod bipolar cell level after 6 weeks of diabetes. A. example traces of light-evoked IPSCs in the same control (A1) or diabetic (A2) dark-adapted cell in response to 30 ms stimuli (gold bars) of $9.5 \cdot 10^5$ photons $\cdot\mu\text{m}^{-2}\cdot\text{s}^{-1}$ before (dark blue/red) and after (light blue/pink) application of SKF. B,C. Average normalized light-evoked IPSC peak amplitude (B) and Q (C) of light-evoked IPSCs recorded after application of SKF in control (light blue squares) and diabetic (pink triangles) cells. All data is normalized to the maximum value recorded for each cell before SKF. D and E. Average

normalized time to peak (D) and D37 (E) values after SKF application in control (light blue squares) and diabetic (pink triangles) cells. SKF caused significant declines in control and diabetic cells for all quantified parameters, but the degree to which it modified responses in both groups was not different. For B and C, control n = 6 and diabetic n = 9. For D and E, control n = 4 and diabetic n = 6. P values reported above each graph are for the 2-way ANOVA main effects of diabetes, accompanied by an * if significant. #,\$ = significant pairwise difference between dark-adapted and SKF-treated states at a specific intensity for control and diabetic groups, respectively. † = significant pairwise difference of SKF-treated responses between control and diabetic groups (SNK post-hoc).

Figure 4.

D1R activation causes similar changes to spontaneous IPSCs in control and diabetic rod bipolar cells. A. Example spontaneous IPSC traces from the same control (A1) and diabetic (A2) cells before (top) and after (bottom) application of SKF. B. Average normalized spontaneous IPSC frequency (B1), amplitude (B2) and decay τ (B3) for control (light blue) and diabetic (pink) rod bipolar cells after SKF. Individual datapoints are shown as white circles (control) and white triangles (diabetic). Average values for each cell were normalized to the average values recorded under dark-adapted conditions. Unity (i.e., no change from

dark-adapted conditions) is represented by a dashed black line. P values shown are for unpaired t-tests between control and diabetic groups. ‡,§ = significantly different via paired t-test compared to dark-adapted conditions for control and diabetic groups, respectively. C1. (Left) Inter-event interval (IEI) histogram for the same control cell in A1 before (dark blue) and after (light blue) application of SKF, normalized to the total number of events in each condition. Arrows denote the average spontaneous IPSC values before and after SKF application. # = significantly different compared to dark-adapted conditions via Kolmogorov-Smirnov (KS) test. (Right) Average inter-event interval cumulative distributions for all control cells ($\pm$ SEM). All cumulative distributions are normalized along the x-axis by the maximum quantity (e.g., spontaneous IPSC IEI) recorded for each cell. C2. Same as C1, but for the diabetic cell shown in A2 (left) and all diabetic cells (right). D-E: Average cumulative amplitude (D) and decay τ (E) histograms for spontaneous IPSCs before (dark blue/red) and after (light blue/pink) application of SKF for all control (D1,E1) and diabetic (D2,E2) cells.

Figure 5.

Light adaptation is not significantly impaired at the rod bipolar cell level after 6 weeks of diabetes. A. example traces of light-evoked IPSCs from the same control (A1, left) or diabetic (A2, right) dark-adapted cells in response to 30 ms stimuli (small gold bars below traces) of $9.5 \cdot 10^5 \text{ photons} \cdot \mu\text{m}^{-2} \cdot \text{s}^{-1}$ before (dark blue/red) and after (light blue/pink) light adaptation. B and C. Average light-evoked IPSC peak amplitude (B) or Q (C) after light adaptation in control (light blue squares) and diabetic (pink triangles) cells. D and E. Normalized time to peak (D) and D37 (E) values for control (light blue squares) and diabetic

(pink triangles) cells after light adaptation. All data was normalized to the maximum value recorded for each cell before light adaptation. For B and C, control n=7 and diabetic n=6. For D and E, control n=5 and diabetic n=4. P values reported above each graph are for the 2-way ANOVA main effects of diabetes. #,\$ = significant pairwise difference between dark-adapted and light-adapted states at a specific intensity for control and diabetic groups, respectively (SNK post-hoc).

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Figure 6.

Light adaptation causes similar changes to spontaneous IPSCs in control and diabetic rod bipolar cells. A. Example spontaneous IPSC traces from the same control (A1) and diabetic (A2) cell before (top) and after (bottom) light adaptation. B. Average normalized spontaneous IPSC frequency (B1), amplitude (B2) and decay τ (B3) for control (light blue) and diabetic (pink) rod bipolar cells after light adaptation. Individual datapoints are shown as white circles (control) and white triangles (diabetic). Average values for each cell were normalized to the average values recorded under dark-adapted conditions. Unity (i.e., no

change from dark-adapted conditions) is represented by a dashed black line. P values shown are for unpaired t-tests between control and diabetic groups. ‡,§ = significantly different via paired t-test compared to dark-adapted conditions for control and diabetic groups, respectively. C1. (Left) Inter-event interval histogram for the same control cell in A1 before (dark blue) and after (light blue) light adaptation, normalized to the total number of events in each condition. Arrows denote the average IEI values before and after light adaptation. # = significantly different compared to dark-adapted conditions via KS test. (Right) Average inter-event interval cumulative distributions for all control cells. C2. Same as C1, but for the diabetic cell shown in A2 (left) and all diabetic cells (right). D-E: Average amplitude (D) and decay τ (E) cumulative histograms for spontaneous IPSCs before (dark blue/red) and after (light blue/pink) light adaptation for all control (D1,E1) and diabetic (D2,E2) cells. All cumulative distributions are normalized along the x-axis by the maximum quantity (e.g., spontaneous IPSC amplitude) recorded for each cell and displayed as average $\pm$ SEM.

Figure 7.

Light adaptation is significantly impaired at the ganglion cell level after 6 weeks of diabetes. A. example traces of light-evoked EPSCs from the same control (A1) or diabetic (A2) dark-adapted cells in response to 30 ms stimuli (gold bars) of $9.5 \cdot 10^3$, $9.5 \cdot 10^4$, and $9.5 \cdot 10^5$ photons $\cdot \mu\text{m}^{-2} \cdot \text{s}^{-1}$ before (dark blue/red) and after (light blue/pink) light adaptation. B and C. Average normalized light-evoked EPSC peak amplitude (B) or Q (C) after light adaptation in control (light blue squares) and diabetic (pink triangles) cells. D and E. Normalized time to peak (D) and D37 (E) values for control (light blue squares) and diabetic (pink triangles)

cells after light adaptation. All data was normalized to the maximum value recorded for each cell. For B and C, control n=9 and diabetic n=11. For D and E, control n=8 and diabetic n=10. P values reported above each graph are for the 2-way ANOVA main effects of diabetes, accompanied by an * if significant. #,\$ = significant pairwise difference between dark-adapted and light-adapted states at a specific intensity for control and diabetic groups, respectively (SNK post-hoc).

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Figure 8.

Light adaptation causes similar changes to spontaneous EPSCs in control and diabetic ON sustained ganglion cells. A: Example spontaneous EPSC traces from the same control (A1) and diabetic (A2) cells before (top) and after (bottom) light adaptation. B: Average normalized spontaneous EPSC frequency (B1), amplitude (B2), and decay τ (B3) for control (light blue) and diabetic (pink) ON sustained ganglion cells after light adaptation. Data for individual control cells are shown as white circles (control) and white triangles (diabetic). Unity (i.e., no change from dark-adapted conditions) is represented by a dashed black line.

Average values for each cell were normalized to the average values recorded under dark-adapted conditions. P values shown are for unpaired t-tests between control and diabetic groups. ‡,§ = significantly different via paired t-test compared to dark-adapted conditions for control and diabetic groups, respectively. C1. (Left) Inter-event interval histogram for the same control cell in A1 before (dark blue) and after (light blue) light adaptation, normalized to the number of events per condition. Arrows denote the average spontaneous EPSC values before and after light adaptation. # = significantly different compared to dark-adapted conditions via KS test. (Right) Average inter-event interval cumulative histograms for all control cells. C2. Same as C1, but for the diabetic cell shown in A2 (left) and all diabetic cells (right). D-E: Average amplitude (D) and decay τ (E) cumulative histograms for spontaneous IPSCs before (dark blue/red) and after (light blue/pink) light adaptation for all control (D1,E1) and diabetic (D2,E2) cells. All cumulative distributions are normalized along the x-axis by the maximum quantity (e.g., spontaneous IPSC amplitude) recorded for each cell. Averages $\pm$ standard errors are denoted by solid lines.

Figure 9.

No evidence of dopaminergic amacrine cell death after 6 weeks of diabetes. A1-2. Example z-stack image of tyrosine hydroxylase staining in the INL of a control (A1) and diabetic (A2) retina, captured with 10x objective. Scale Bar = 200 μm. A3-4: Same as A1-2, but with 40x objective; scale bar = 25 μm. B. Average tyrosine hydroxylase positive cell density (B1), soma size (B2), and soma intensity (B3) for control (blue) and diabetic (red) retinas. P values are reported for unpaired t-tests between diabetic and control groups. No significant differences were found.

Figure 10.

No evidence of retinal cell loss in the GCL or amacrine cell loss in the INL after 6 weeks of diabetes. A: Identification of ganglion and displaced amacrine cell populations in the GCL of a control retina. Ganglion cell (A1) and displaced amacrine cell (A2) populations were selectively labeled with antibodies against RBPM5 (magenta) and GAD65/67 (green), respectively. The overlay (A3) demonstrates the lack of any substantial co-labeling, allowing discrimination of ganglion vs. displaced amacrine cell identity. B. Same as A, but in a diabetic retina. C. Cell counts of control and diabetic mice show that there is no significant

difference in ganglion cell number (C1), displaced amacrine cell number (C2) or total cell number (C3) in the GCL after 6 weeks of diabetes. D. Representative images of the glycinergic amacrine cell population (D1) and GABAergic amacrine cell population (D2) in a control retina, selectively labeled with GlyT1 antibody (blue) and GAD65/67 antibodies (green), respectively. The overlay (D3) shows no overlapping signal, demonstrating excellent immunohistochemical discrimination. E. Same as in D, but in a diabetic retina. F. Cell counts of control and diabetic mice show that there is no significant difference in glycinergic (F1), GABAergic (F2), or total (F3) amacrine cell number in the INL after 6 weeks of diabetes. All scale bars = 50 μ m. P values for unpaired t-tests between control and diabetic.

Table 1.

Primary Antibodies used in immunohistochemistry.

Antibody name	Abbreviation	Dilution	Company	Catalog #	Reference
mouse anti-Tyrosine Hydroxylase	TH	1:2000	Millipore	MAB5280	(Vuong et al., 2015)
rabbit anti-Cleaved Caspase-3	Caspase-3	1:300	Cell Signaling	#9661	(Chen and Nathans, 2007)
mouse anti-Glutamic Acid Decarboxylase 65 IgG2a	GAD 65	1:1000	deposited to the Developmental Studies Hybridoma Bank by Gottlieb	#GAD-6	(Essrich et al., 1998; Shibasaki et al., 2007)
mouse anti-Glutamic Acid Decarboxylase 67 IgG2a	GAD 67	1:1000	Millipore	#MAB5406	(Perez de Sevilla Muller et al., 2017);
goat anti-Glycine Transporter 1	GlyT1	1:50	Santa Cruz Biotech	#sc-16703	(Jo et al., 2018)
guinea pig anti-Ribosome Binding Protein Multiple Splice	RBPMs	1:500	PhosphoSolutions	#1832-RBPMs	(Rodriguez et al., 2014)

Table 2.

Effects of the dopamine D1 receptor agonist SKF-38393 on spontaneous IPSC parameters in control and diabetic rod bipolar cells.

Spontaneous IPSC parameter	Control (SKF)	Diabetic (SKF)	Significance control vs. diabetic
Frequency (Norm. to Dark)	$0.315 \pm 0.093^{***}$ (n=6)	$0.364 \pm 0.206^*$ (n=6)	0.832
Amplitude (Norm. to Dark)	0.926 ± 0.133 (n=6)	1.028 ± 0.270 (n=4)	0.715
Decay τ (Norm. to Dark)	1.185 ± 0.098 (n=6)	1.053 ± 0.064 (n=4)	0.345

*
p<0.05

p<0.001, SKF vs. dark

Table 3.

Effects of light adaptation (LA) on spontaneous IPSC parameters in control and diabetic rod bipolar cells.

Spontaneous IPSC parameter	Control (LA)	Diabetic (LA)	Significance control vs. diabetic
Frequency (Norm. to Dark)	0.295 ± 0.061 *** (n=5)	0.322 ± 0.169 * (n=5)	0.887
Amplitude (Norm. to Dark)	0.734 ± 0.129 (n=5)	1.059 ± 0.231 (n=4)	0.236
Decay τ (Norm. to Dark)	1.020 ± 0.158 (n=5)	0.946 ± 0.134 (n=4)	0.738

*
p<0.05

p<0.001, LA vs. dark

Table 4.

Effects of light adaptation (LA) on spontaneous EPSC parameters in control and diabetic ONs ganglion cells.

Spontaneous EPSC parameter	Control (LA)	Diabetic (LA)	Significance control vs. diabetic
Frequency (Norm. to Dark)	$0.614 \pm 0.148^*$ (n=10)	$0.419 \pm 0.117^{***}$ (n=10)	0.314
Amplitude (Norm. to Dark)	1.001 ± 0.119 (n=10)	0.885 ± 0.103 (n=10)	0.470
Decay τ (Norm. to Dark)	1.082 ± 0.127 (n=10)	1.089 ± 0.166 (n=10)	0.973

*
p<0.05***
p<0.001, LA vs. dark

Table 5.

Dopaminergic amacrine cell quantification in control and diabetic retinas.

	Control	Diabetic	Significance control vs. diabetic
Cell density (cells/mm ²)	44.023 ± 0.945 (n=7)	43.290 ± 0.546 (n=6)	0.535
Soma size (μm ²)	127.560 ± 1.982 (n=4)	133.109 ± 2.387 (n=4)	0.124
Soma intensity (Relative Units)	11046.006 ± 1930.240 (n=3)	11074.104 ± 1524.780 (n=3)	0.994

Table 6.

Numbers of immunolabeled neurons in different retinal layers.

Cell type	Control	Diabetic	Significance control vs. diabetic
Neurons in GCL	11,093 $\pm$ 206.6 cells/mm ² (n = 14 mice)	11,260.7 $\pm$ 153.7 cells/mm ² (n=12 mice)	$p=0.533$
Ganglion cells in GCL	6861.8 $\pm$ 167.1 cells/mm ² (n = 3 mice)	6943.4 $\pm$ 160.1 cells/mm ² (n = 4 mice)	$p = 0.629$
Displaced amacrine cells in GCL	3035.6 $\pm$ 153.1 cells/mm ² (n = 14 mice)	3177.3 $\pm$ 123.2 cells/mm ² (n = 12 mice)	$p = 0.488$
GABAergic amacrine cells INL	7559.9 $\pm$ 120.2 cells/mm ² (n = 14 mice)	7362.6 $\pm$ 198.8 cells/mm ² (n = 12 mice)	$p = 0.389$
glycinergic amacrine cells INL	5679.2 $\pm$ 75.7 cells/mm ² (n = 7 mice)	5717.3 $\pm$ 202.1 cells/mm ² (n = 6 mice)	$p = 0.855$
Total Amacrine Cells in INL	13,247.0 $\pm$ 157.9 (n=7)	13152.7 $\pm$ 135.3 (n=6)	$p = 0.665$