

# Dopamine Deficiency Mediates Early Rod-Driven Inner Retinal Dysfunction in Diabetic Mice

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**PURPOSE.** Electroretinograms (ERGs) are abnormal in diabetic retinas before the appearance of vascular lesions, providing a possible biomarker for diabetic vision loss. Previously, we reported that decreased retinal dopamine (DA) levels in diabetic rodents contributed to early visual and retinal dysfunction. In the current study, we examined whether oscillatory potentials (OPs) could serve as a potential marker for detecting early inner retinal dysfunction due to retinal DA deficiency.

**METHODS.** Retinal function was tested with dark-adapted ERGs, taken at 3, 4, and 5 weeks after diabetes induction with streptozotocin. Electrical responses were analyzed and correlations were made with previously reported retinal DA levels. The effect of restoring systemic DA levels or removing DA from the retina in diabetic mice on OPs was assessed using L-3,4-dihydroxyphenylalanine (L-DOPA) treatments and retina-specific tyrosine hydroxylase (*Tb*) knockout mice (rTHKO), respectively.

**RESULTS.** Diabetic animals had significantly delayed OPs compared to control animals in response to dim, but not bright, flash stimuli. L-DOPA treatment preserved OP implicit time in diabetic mice. Diabetic rTHKO mice had further delayed OPs compared to diabetic mice with normal retinal *Tb*, with L-DOPA treatment also providing benefit. Decreasing retinal DA levels significantly correlated with increasing OP delays mediated by rod pathways.

**CONCLUSIONS.** Our data suggest that inner retinal dysfunction in early-stage diabetes is mediated by rod-pathway deficits and DA deficiencies. OP delays may be used to determine the earliest functional deficits in diabetic retinopathy and to establish an early treatment window for DA therapies that may prevent progressive vision loss.

Keywords: diabetic retinopathy, dopamine, oscillatory potentials, electroretinogram

Diabetic retinopathy (DR) is a vision-threatening complication of diabetes mellitus (DM). After considering population growth and aging, DM in adults has nearly quadrupled in the last 30 years<sup>1</sup> and is predicted to increase 54% by 2030.<sup>2</sup> Concurrently, the incidence of vision loss due to DR is expected to nearly double between 2010 and 2050.<sup>1,3-5</sup>

A growing body of literature suggests that neuronal dysfunction in the retina is observable in early diabetes, before any vascular abnormalities are present in the eye.<sup>6-10</sup> Changes in retinal thickness, alterations in color vision and contrast sensitivity, and decreased electroretinogram (ERG) responses have been detected in diabetic patients with no or minimal retinopathy.<sup>9,11-22</sup> In diabetic animal models, altered glial cell activities,<sup>23-25</sup> loss of retinal neurons, downregulation of synaptic proteins,<sup>26</sup> and attenuated electrical responses of the inner retinal neurons<sup>8,27-33</sup> have been found. Collectively, these studies strongly suggest negative effects of hyperglycemia in the sensory neurons of the retina, and emphasize the need to

elucidate the underlying mechanisms to find novel treatment options to preserve vision earlier in the disease.

Chronic hyperglycemia appears to alter neuronal function in part by reducing the synthesis of dopamine (DA), a key neuromodulator in the retina and brain.<sup>34</sup> Alterations to DA metabolism and synthesis in retinal neurons have been documented in streptozotocin (STZ)-induced hyperglycemic rats as early as 3 weeks post STZ.<sup>35,36</sup> In addition, loss of dopaminergic amacrine cells was reported in animal models of both chemically and spontaneously induced diabetes 6 months after the onset of disease.<sup>37</sup> Dopamine dysregulation in the diabetic rat retina has been shown to have functional consequences, including delayed b-wave latencies and reduced spatial frequency and contrast sensitivity thresholds in the early stages of disease.<sup>35</sup> Further implicating DA's role in neural dysfunction during diabetes, visual function in these same animals could be partially preserved with the treatment of L-3,4-dihydroxyphenylalanine (L-DOPA), a precursor to DA.<sup>35</sup>



Oscillatory potentials (OPs), high-frequency oscillations on the leading edge of the ERG b-wave,<sup>38,39</sup> have shown consistent delays in diabetic subjects<sup>6-8</sup> and in diabetic animals even prior to development of vascular abnormalities.<sup>27,29-32</sup> We have previously reported rod-mediated OP delays in hyperglycemic rats as early as 4 weeks post STZ.<sup>33</sup> Since these small wavelet OPs are generated by the inner retina,<sup>38</sup> these early deficits suggest a susceptibility of the inner retina to diabetic insult.

The aim of the current study was to determine whether the early changes in OP latency due to diabetes are attributable to DA deficiencies affecting inner retinal function. Here we examined the temporal appearance of OP delays in early-stage DR in STZ-induced diabetic mice and evaluated the effects of DA therapy using L-DOPA or DA deficiency using a retinal-specific tyrosine hydroxylase (*Tb*) knockout mouse model to evaluate the contributions of DA signaling on OP delays.

## METHODS

### Animals

Albino CD-1 and pigmented C57BL mice were obtained from Charles River Laboratories (Wilmington, MA, USA) and Jackson Laboratories (Bar Harbor, ME, USA), respectively, and housed in the animal facility at the Atlanta Veterans Affairs Medical Center (Decatur, GA) under a 12:12 (light:dark) hour cycle with food and water ad libitum. All procedures were approved by the Institutional Animal Care and Use Committee of the Atlanta Veterans Affairs Medical Center and performed in full accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

DM CD-1 mice: Diabetic mice were used to find ERG deficits with hyperglycemia. Diabetic mice (DM;  $n = 11$ ) received STZ (200 mg/kg; Sigma-Aldrich Corp., St. Louis, MO, USA) while control mice (CTRL;  $n = 11$ ) received equal volumes of citrate buffer. STZ was dissolved in citrate buffer mixed in 50% glucose (pH 4.0) and immediately injected intraperitoneally (IP). Blood glucose (BG) levels were measured using a handheld glucose meter (Freestyle; Abbott, Abbott Park, IL, USA) with blood collected from the tail vein. DM was confirmed with two successive measurements of BG greater than 200 mg/dL BG, and body weights (BW) were checked every other day for the first week and weekly thereafter until the end of the study. Upon two successive measurements of weight reduction in hyperglycemic animals, insulin (1 mL; Lantus Sanofi-Aventis, Bridgewater, NJ, USA) was injected intraperitoneally to prevent further weight loss. The low-dose insulin injection did not cause hypoglycemia. Retinas from a subset of these mice (CTRL,  $n = 8$ ; DM,  $n = 6$ ) were used for DA analysis, as described below.

L-DOPA-treated and *Tb*-deficient mice: To restore retinal DA levels, diabetic mice (genotype information below) were given daily IP injections of vehicle only or L-DOPA (10 mg/kg; Sigma-Aldrich Corp.) dissolved in 0.1% ascorbic acid in saline. L-DOPA was made fresh daily with treatments beginning the week after STZ injections and lasting until 6 weeks post hyperglycemia. All injections occurred 4 to 8 hours after light onset. ERGs were measured 30 to 60 minutes after L-DOPA or vehicle injection.

Tyrosine hydroxylase (TH) is an enzyme critical for the conversion of tyrosine to L-DOPA for the synthesis of DA. To determine the effect of chronic reduction of retinal DA on inner retinal dysfunction in diabetes, we used retina-specific *Tb* gene deletion. Commercially available *Cbx10-Cre* mice (Jackson Laboratories) were crossed with mice expressing a floxed *Tb* allele on C57BL background, as previously described.<sup>40</sup>

Offspring with *Cbx10-Cre:Tb<sup>loxP/loxP</sup>* genotype result in an 85% reduction in retinal DA,<sup>41</sup> and were designated as rTHKO. Mice with the *Tb<sup>loxP/loxP</sup>* genotype were referred to as TH<sub>ctrl</sub>. Diabetes was induced with five daily injections of low-dose STZ (50 mg/kg) (DM rTHKO + Veh,  $n = 8$ ; DM TH<sub>ctrl</sub> + Veh,  $n = 15$ ; TH<sub>ctrl</sub>,  $n = 15$ ). The baseline BG levels of rTHKO mice were normal ( $181.8 \pm 7.75$  mg/dL,  $n = 17$ ) versus TH<sub>ctrl</sub> ( $180.6 \pm 6.5$  mg/dL,  $n = 19$ ; *t*-test *P* = nonsignificant).

To determine the effect of DA treatment on inner retinal function in rTHKO mice, a subset of mice (DM TH<sub>ctrl</sub> + L-DOPA,  $n = 15$ ; DM rTHKO + L-DOPA,  $n = 8$ ) were given injections of L-DOPA (IP, 10 mg/kg; Sigma-Aldrich Corp.) as described above. Half of the TH<sub>ctrl</sub> mice were given L-DOPA and half given vehicle, but there was no statistical difference between the groups so they were combined for analyses. Average BG and BW for these mice can be found in Aung et al.<sup>35</sup>

Animals used in the experiments were followed for 6 weeks post STZ. Mice involved in the DA study were killed by cervical dislocation in order to measure retinal DA using HPLC without interference of anesthesia, while all others were euthanized with an overdose of pentobarbital.

### Measuring Retinal Function With Scotopic Full-Field ERG

Full-field scotopic ERGs were measured at baseline (data not shown) and 3, 4, and 5 weeks post STZ using a commercial ERG system (UTAS E-3000; LKC Technologies, Gaithersburg, MD, USA). After overnight dark adaptation, animals were anesthetized under red light with ketamine (80 mg/kg) and xylazine (16 mg/kg); corneas were anesthetized (0.5% tetracaine HCl), and pupils were dilated (1.0% cyclopentolate HCl and 1.0% tropicamide). Body temperature was maintained at 37°C with a heating pad. Custom-made DTL fiber electrodes<sup>42-45</sup> were used as recording electrodes, contacting the cornea through a thin layer of methylcellulose (Refresh Celluvisc; Allergan, Irvine, CA, USA), while platinum needle electrodes (Grass Technologies, West Warwick, RI, USA) were inserted in the cheeks and tail as reference and ground, respectively. Ganzfeld flash stimuli ranging from  $-3.4$  to  $1.4$  log cd s/m<sup>2</sup>, with interstimulus intervals ranging from 4 to 65 seconds, were used to elicit a retinal response. Responses were differentially amplified (1-1500 Hz) with a recording length of 250 ms and a sampling rate of 2000 Hz (UTAS Bigshot; LKC Technologies). After the recording, animals were given an IP injection of yohimbine (2.0 mg/kg; Lloyd Laboratories, Shenandoah, IA, USA) for recovery from anesthesia and prevention of corneal ulcers.<sup>46</sup> STZ-treated mice were given a subcutaneous bolus of 0.9% saline solution for hydration after the recording.

ERG data analysis was performed on one eye of each animal. Four animals were excluded from the study, due to death ( $n = 1$ ), absence of mixed rod- and cone-mediated ERG response ( $n = 2$ ), and failed outlier test ( $n = 1$ ). Amplitudes and implicit times of a- and b-wave and OPs were analyzed, as previously described.<sup>47</sup> Inner retinal function was examined by extracting OPs from the ERG waveforms<sup>38,48,49</sup> using a bandpass filter setting of 75 to 500 Hz (EM for Win version 8.1.2, LKC Technologies). Flashes within the rod-dominated dim luminance range<sup>8,33</sup> of  $-3.02$  and  $-1.8$  log cd s/m<sup>2</sup> were compared to a bright flash of  $0.61$  log cd s/m<sup>2</sup> to compare the inner retinal function dominated by rod pathways versus mixed rod and cone pathways, respectively.<sup>50,51</sup>

### Dopamine Analysis

After euthanasia at 6 weeks post STZ, retinas were taken from a subset of mice ( $n = 14$ ) and DA levels were analyzed, as

TABLE. Blood Glucose and Weight Measurements in Diabetic and Control Mice

Timepoints		Body Weight, g				Blood Glucose, mg/dL			
		Control, n = 11		Diabetic, n = 11		Control, n = 11		Diabetic, n = 11	
Age	Post STZ	Mean	±SEM	Mean	±SEM	Mean	±SEM	Mean	±SEM
P33	Wk 0	26.3	0.45	26.6	0.59	187.1	9.76	185.7	9.29
P38	Wk 1	39.8	0.64	28	1.03	154.3	7.51	344.3*	30.08
P80	Wk 6	37.8	1.08	30.9*	0.71	138.4	5.95	507.7*	31.01

P, postnatal age (days).

\*  $P < 0.001$ , 2-way repeated-measures ANOVA, Holm-Sidak post hoc diabetic versus control.

previously described.<sup>35</sup> Retinas were flash-frozen and stored at  $-20^{\circ}\text{C}$ . Ion-pair reverse-phase HPLC with coulometric detection was used to measure DA levels. Frozen retinas were homogenized in a 0.2 N  $\text{HClO}_4$  solution with 0.01% sodium meta-bisulfite and 25 ng/mL 3,4-dihydroxybenzylamine hydrobromide as an internal standard. Samples were then centrifuged, and supernatant fractions were separated on a HPLC column (Ultrasphere ODS; Hichrom, Berkshire, UK). DA was

quantified based on a standard curve from 2 to 20 ng/mL, and measured as picograms per retina.

### Statistical Analysis

Statistical analyses were performed with repeated-measures ANOVAs (Holm-Sidak post hoc analyses, SigmaStat 3.5; Systat Software, Chicago, IL, USA) when comparing the diabetic and control groups across time. One-way ANOVAs with Holm-Sidak

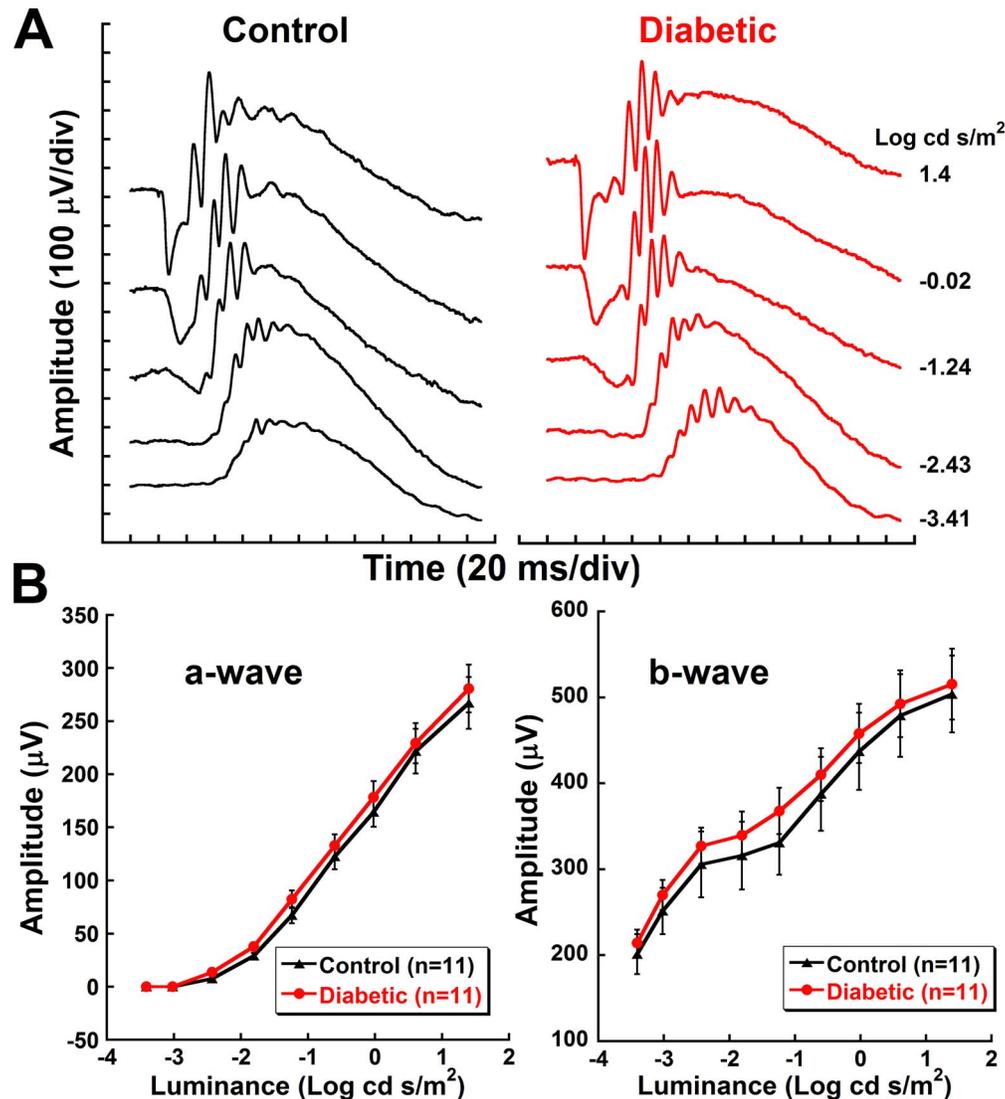
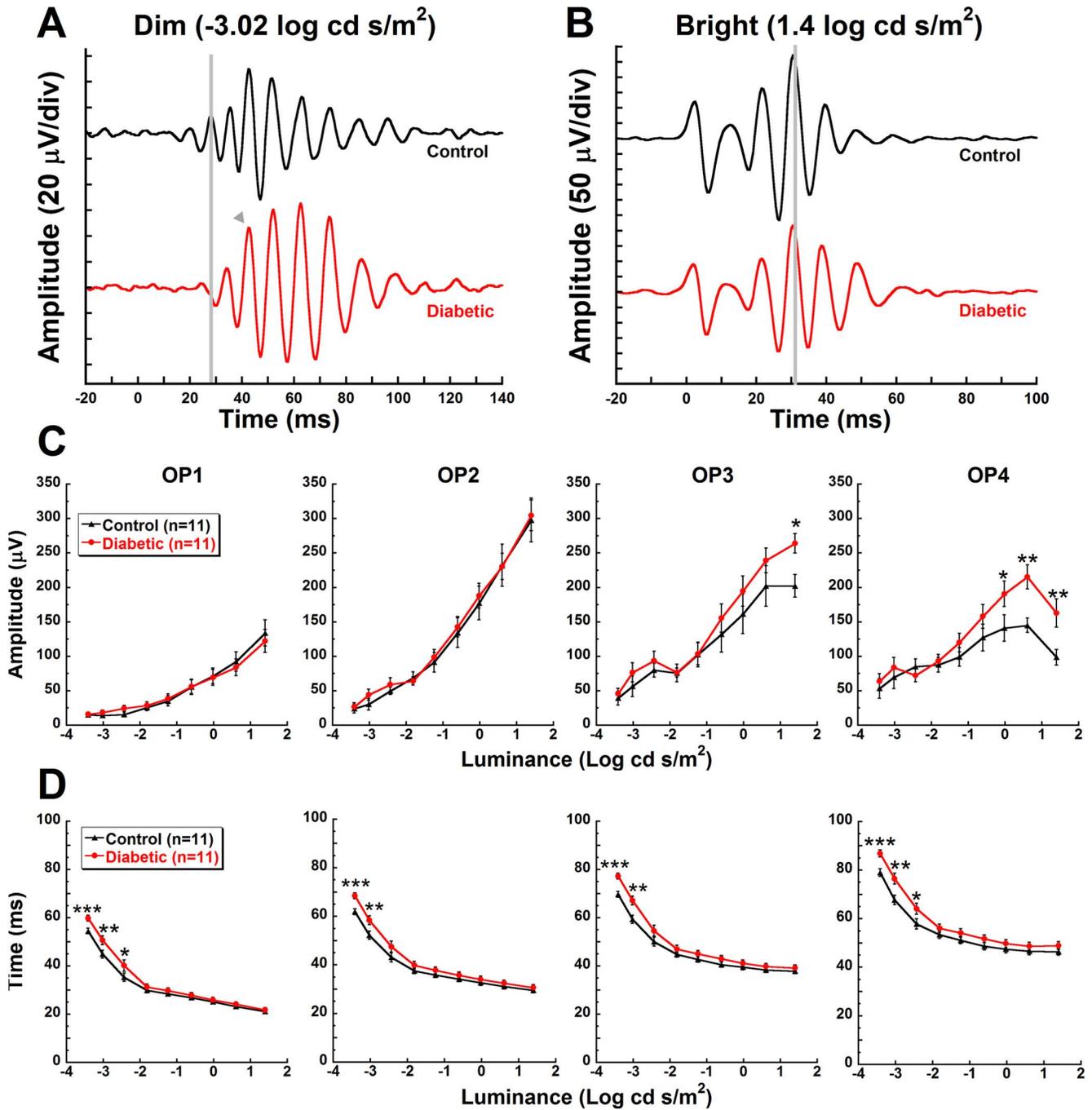


FIGURE 1. No differences in ERG a- and b-wave responses at 5 weeks post STZ. (A) Representative raw ERG waveforms of control and diabetic animals at 5 weeks post STZ. (B) Mean ( $\pm\text{SEM}$ ) amplitudes of a- and b-waves at 5 weeks post STZ plotted against increasing flash stimuli.



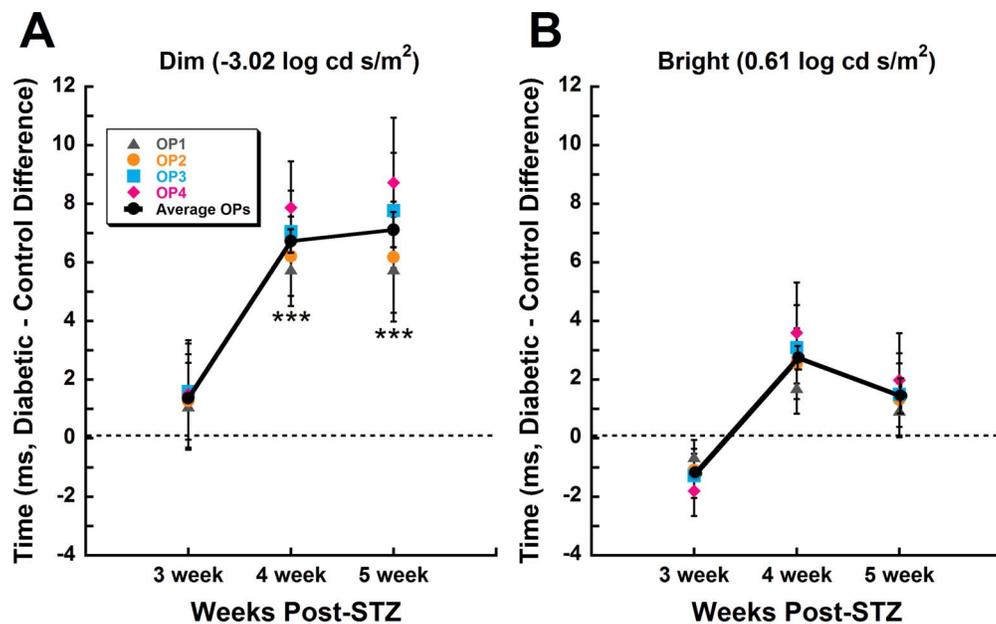
**FIGURE 2.** Delayed implicit times of rod-driven OPs in diabetic animals. Representative OP waveforms from control and diabetic mice in response to a representative (A) dim ( $-3.02 \log \text{cd s/m}^2$ ) or (B) bright flash ( $1.4 \log \text{cd s/m}^2$ ). Flash onset was at 0 ms. The *gray lines* indicate OP2 for the control animal and the *gray arrowhead* indicates a delayed OP2. OP amplitudes (C) and implicit times (D) (mean  $\pm$  SEM) of OP1, OP2, OP3, and OP4 at 5 weeks post STZ are plotted against flash stimuli. Asterisks represent Holm-Sidak post hoc analysis for individual steps: \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

post hoc analyses using the rough false discovery rate (RFDR) method for alpha level adjustment [calculated as  $p^{*(\# \text{tests} + 1)} / (2^{*(\# \text{tests})})$ ] were used for analysis of L-DOPA-treated mice.<sup>35</sup> All four OPs generated similar results, so OP2 was chosen for presentation here. Pearson's correlation test was used to calculate the correlation coefficient between the retinal DA level (pg/retina) and OP2 implicit time (ms) at dim ( $-3.02 \log \text{cd s/m}^2$ ) and bright ( $0.61 \log \text{cd s/m}^2$ ) flash stimuli. All figures show mean values with error bars representing SEM. Reported  $P$  values represent Holm-Sidak post hoc results.

## RESULTS

### Blood Glucose and Body Weight of STZ-Injected CD-1 Mice

As expected, mice injected with STZ became hyperglycemic ( $344 \pm 30 \text{ mg/dL}$ ) compared to controls ( $154 \pm 7.5 \text{ mg/dL}$ ) within a week, and their BG levels remained elevated ( $507 \pm 31.01 \text{ mg/dL}$ ) to the end of the study (Table,  $P < 0.001$ ). Mice



**FIGURE 3.** Rod-driven stimuli reveal selective delays in OP implicit time across diabetes progression, unlike mixed rod-cone stimuli. Mean ( $\pm$ SEM) OP1 through OP4 implicit time differences of diabetic relative to control animals over the duration of diabetes show progressive delays in response to (A) dim ( $-3.02 \log \text{cd s/m}^2$ ), but not (B) bright ( $0.61 \log \text{cd s/m}^2$ ) flash stimuli. Colored symbols represent each OP, and the solid line with black circles represents the average of all four OPs. Asterisks represent Holm-Sidak post hoc analysis for individual weeks,  $***P < 0.001$ .

injected with STZ failed to gain weight over time, but remained relatively stable over the 5 weeks of hyperglycemia.

#### ERG Shows OP Delays in Diabetic CD-1 Mice After 4 to 5 Weeks Post STZ

Representative ERG recordings illustrated no discernable differences in a- and b-waveforms between the two groups at this early stage of diabetes (Fig. 1A). No significant differences were found in the a- and b-wave amplitudes (Fig. 1B) or implicit times (data not shown).

No differences were observed in any of the OP amplitudes at 3 or 4 weeks post STZ (data not shown). Similarly, at 5 weeks post STZ, OP1 and OP2 amplitudes were indistinguishable between the groups. However, OP3 and OP4 amplitudes were significantly higher in diabetic animals in response to brighter flash stimuli (Fig. 2C). OP3 showed a 31% increase in amplitude in response to  $1.4 \log \text{cd s/m}^2$  stimuli ( $P = 0.02$ ). OP4 showed a 35% increase to  $-0.02 \log \text{cd s/m}^2$  stimuli ( $P = 0.02$ ), a 48% increase to  $-0.61 \log \text{cd s/m}^2$  stimuli ( $P = 0.002$ ), and a 65% increase to  $1.4 \log \text{cd s/m}^2$  stimuli (Fig. 2C,  $P = 0.004$ ).

In contrast to differential changes in amplitudes across the OPs, implicit times of all four OPs were uniformly delayed by hyperglycemia at 5 weeks post STZ (Fig. 2D). OP implicit times were significantly delayed at 4 weeks (data not shown) and 5 weeks post STZ from 10% to 12% for OP1 through OP4 (Fig. 2D,  $P < 0.05$  to  $P < 0.001$ ). Note that the magnitude of the delay was greatest in response to the dimmest flash stimuli.

Rod-dominated signaling was the most impacted by diabetes. When diabetic versus control differences in all OP implicit times were averaged together and compared between representative dim ( $-3.02 \log \text{cd s/m}^2$ ) and bright ( $0.61 \log \text{cd s/m}^2$ ) stimuli at 3, 4, and 5 weeks post STZ, only responses to dim stimuli showed significant delays in the diabetic animals at week 4 (Fig. 3A,  $P < 0.001$ ) and week 5 (Fig. 3A,  $P < 0.001$ ).

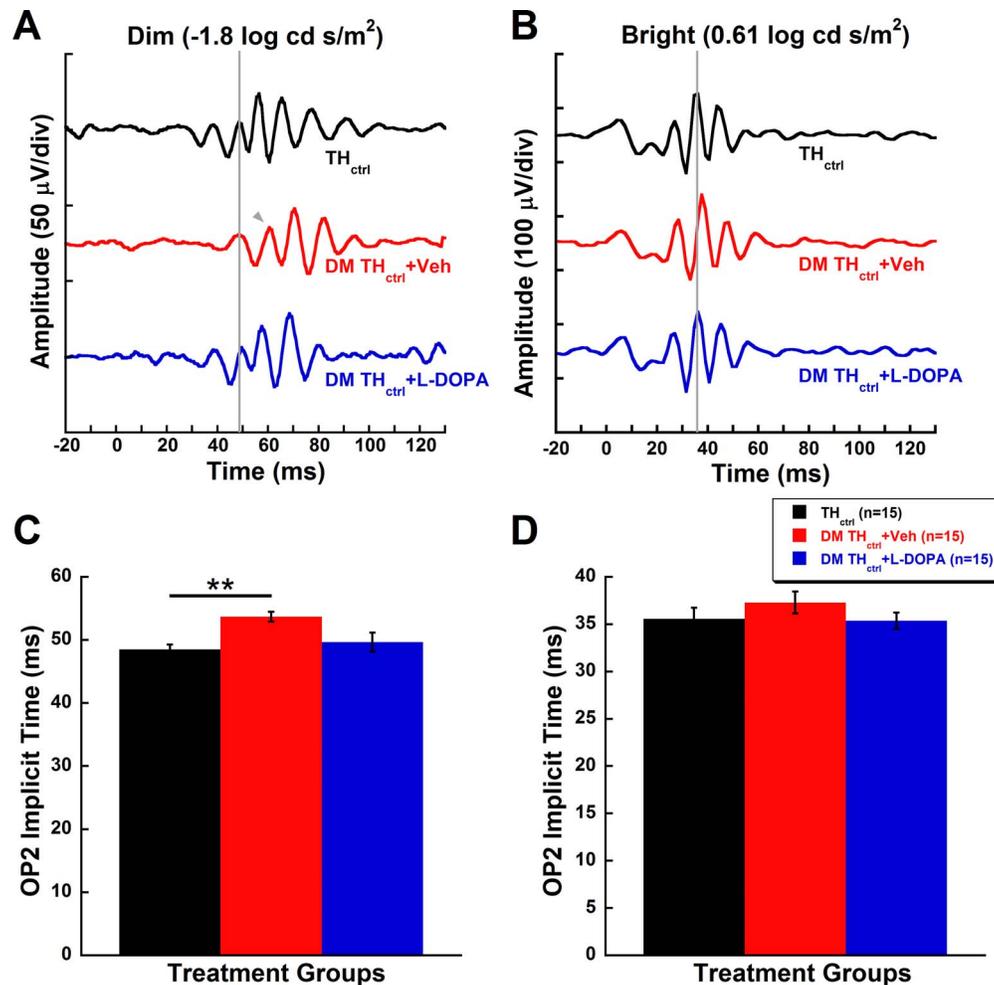
#### Diabetes-Induced OP Delays Are Ameliorated by L-DOPA Treatment

Similar to the data shown in Figure 2, DM TH<sub>ctrl</sub> + Veh animals showed an 11% delay in OP2 implicit times compared to TH<sub>ctrl</sub> animals in response to dim flash stimuli (Figs. 4A, 4C;  $-1.8 \log \text{cd s/m}^2$ ,  $P = 0.008$ ). Treatment with L-DOPA prevented the OP delay induced by hyperglycemia. DM TH<sub>ctrl</sub> + L-DOPA animals were statistically indistinguishable from TH<sub>ctrl</sub> mice (Figs. 4A, 4C,  $P = 0.5$ ), as shown by the representative OP response (gray vertical line marks OP2 in Fig. 4A). OP2 implicit times for DM TH<sub>ctrl</sub> + Veh and DM TH<sub>ctrl</sub> + L-DOPA mice in response to bright flash stimuli did not reach statistical significance (Figs. 4B, 4D).

#### Retinal Dopamine Depletion Elicits OP Delays That Can Be Partially Rescued by L-DOPA

While the DM TH<sub>ctrl</sub> + Veh mice showed a selective delay in response to only dim flash stimuli ( $P = 0.02$ , similar to the CD-1 mice in Figs. 2 and 3), deletion of retinal *Tb* had a deleterious effect on inner retinal function in DM mice in response to both dim and bright flashes. The implicit time of OP2 was 18% delayed in DM rTHKO + Veh mice compared to TH<sub>ctrl</sub> mice for dim flash stimuli (Fig. 5C,  $P = 0.003$ ) and 20% delayed for bright flash stimuli (Fig. 5D,  $P < 0.004$ ). For mixed rod- and cone-mediated responses, the *Tb* knockout produced a delay more extreme compared to diabetes alone. The reduction in retinal Th produced 14% delays in OP2 implicit times for DM rTHKO + Veh animals compared to DM TH<sub>ctrl</sub> + Veh animals in response to bright flash stimuli (Fig. 5D,  $P < 0.03$ ).

L-DOPA treatment restored rod-driven function in DM rTHKO + L-DOPA mice (Fig. 5C) to the same level as the TH<sub>ctrl</sub> group ( $P = 0.54$ ). Interestingly, the treatment did not rescue mixed rod- and cone-driven function. The treatment effect of L-DOPA in DM rTHKO + L-DOPA animals was not statistically significant under the bright flash stimulus (Fig. 5D).



**FIGURE 4.** L-DOPA treatment reverses the rod-driven inner retinal dysfunction in diabetic animals. (A, B) Representative raw waveforms of TH<sub>ctrl</sub>, DM TH<sub>ctrl</sub> + Veh, and DM TH<sub>ctrl</sub> + L-DOPA animals and their (C, D) OP2 implicit times (mean  $\pm$  SEM) at 5 weeks post STZ in response to representative dim ( $-1.8 \log \text{cd s/m}^2$ ; A, C) and bright ( $0.61 \log \text{cd s/m}^2$ ; B, D) flash stimuli. *Gray lines* indicate the onset of OP2 in a representative control animal. The *gray arrow* indicates the onset of OP2 when it is significantly delayed in comparison to the control animal. (C) A significant delay ( $P = 0.008$ ) is seen in OP2 implicit times of DM TH<sub>ctrl</sub> + Veh under dim flash stimulus. Treatment with L-DOPA in diabetic animals ameliorated the functional deficit to a level indistinguishable from the TH<sub>ctrl</sub> group. (D) Bright flash stimulus did not reveal any significant differences among the groups. *Asterisks* represent Holm-Sidak post hoc analysis with RFDR correction: \*\* $P < 0.01$ .

### Lower Retinal Dopamine Levels Correlate With Greater OP Delays

Next, we determined if decreased retinal DA was associated with inner retinal dysfunction, as measured with OP implicit time. A Pearson's correlation test was performed on the OP responses from control and diabetic animals with retinal DA levels from these same mice, as previously reported<sup>35</sup> (Fig. 6). The correlation coefficient was found for the total sample size (inclusive of diabetic and control animals). In response to dim flash stimuli ( $-3.02 \log \text{cd s/m}^2$ ), delays in OP2 implicit time significantly correlated with decreasing retinal DA level ( $R = -0.552$ ,  $P = 0.04$ ; Fig. 6A). In contrast, a nonsignificant correlation ( $R = -0.31$ ;  $P = 0.28$ ) was observed between the DA level and OP2 implicit times at brighter flash luminance ( $0.61 \log \text{cd s/m}^2$ ; Fig. 6B).

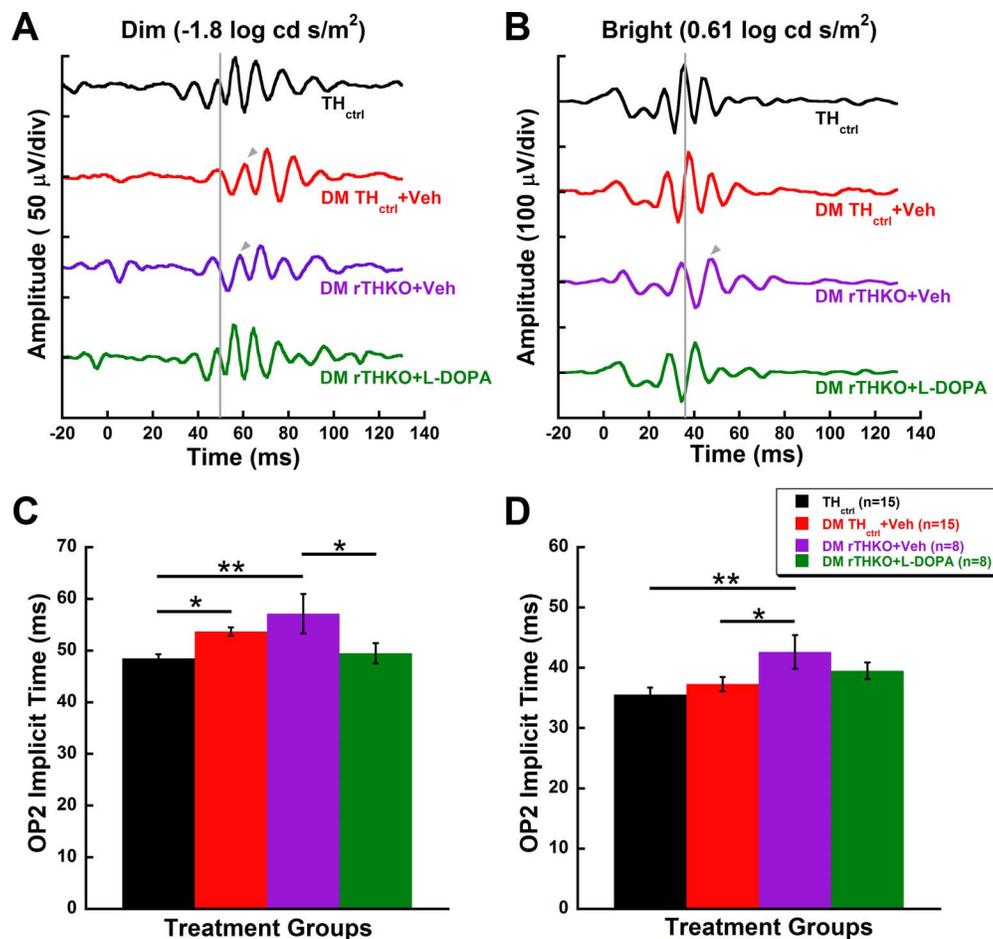
### DISCUSSION

This study showed that inner retinal function, as measured by OP latency, is impacted by diabetes early in the disease, in agreement with previous work.<sup>6-9</sup> Significant reduction of

retinal DA using diabetic rTHKO mice resulted in even greater OP delays. L-DOPA effectively ameliorated rod-dominated functional deficits of the inner retina seen with diabetes and the rTHKO model. The OP delay was mostly limited to dim flash stimuli and correlated with reduced retinal DA levels. Our results indicate that chronic hyperglycemia alters rod-driven inner retinal signal transmission, which is likely mediated by changes in retinal DA levels.

### Inner Retinal Neurons Are Most Susceptible to Early Diabetes

The current study highlights the rapid onset of inner retinal changes with diabetes, which suggests greater vulnerability of inner retinal cell types to dysregulation by chronic hyperglycemia. Delayed OP implicit times have consistently been recognized as indicators of early inner retinal dysfunction in experimental diabetic animal models.<sup>27,29-32</sup> Similarly, observations of altered OPs have been reported in diabetic patients without DR,<sup>6,7</sup> in early DR without detectable vascular abnormalities,<sup>7,9</sup> and in late DR.<sup>9,52</sup> While reductions in OP amplitude<sup>7,9,52</sup> and delays in implicit time<sup>6,7,9</sup> have been



**FIGURE 5.** Diabetic mice lacking retinal *Th* show larger inner retinal dysfunction, of which the rod-driven function can be ameliorated with L-DOPA. (A, B) Representative raw waveforms and (C, D) implicit time (mean  $\pm$  SEM) of TH<sub>ctrl</sub>, DM TH<sub>ctrl</sub> + Veh, DM rTHKO + Veh, and DM rTHKO + L-DOPA animals at 5 weeks post STZ in response to (A, C) dim ( $-1.8 \log \text{cd s/m}^2$ ) and (B, D) bright ( $0.61 \log \text{cd s/m}^2$ ) flash stimuli. Suppression of retina-specific *Th* significantly increased OP2 implicit times compared to the control group in both dim (C;  $P = 0.003$ ) and bright (D;  $P = 0.004$ ) stimuli. However, L-DOPA treatment only protected the OP2 implicit time for the dim flash stimulus (C;  $P = 0.02$ ). Gray lines indicate the onset of OP2 in a representative control animal. Gray arrows indicate the onset of OP2 whenever it is delayed in comparison to the control animal. Asterisks represent Holm-Sidak post hoc analysis with RFDR correction: \* $P < 0.03$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . The waveforms and data for TH<sub>ctrl</sub> and DM TH<sub>ctrl</sub> + Veh are the same as for Figure 4.

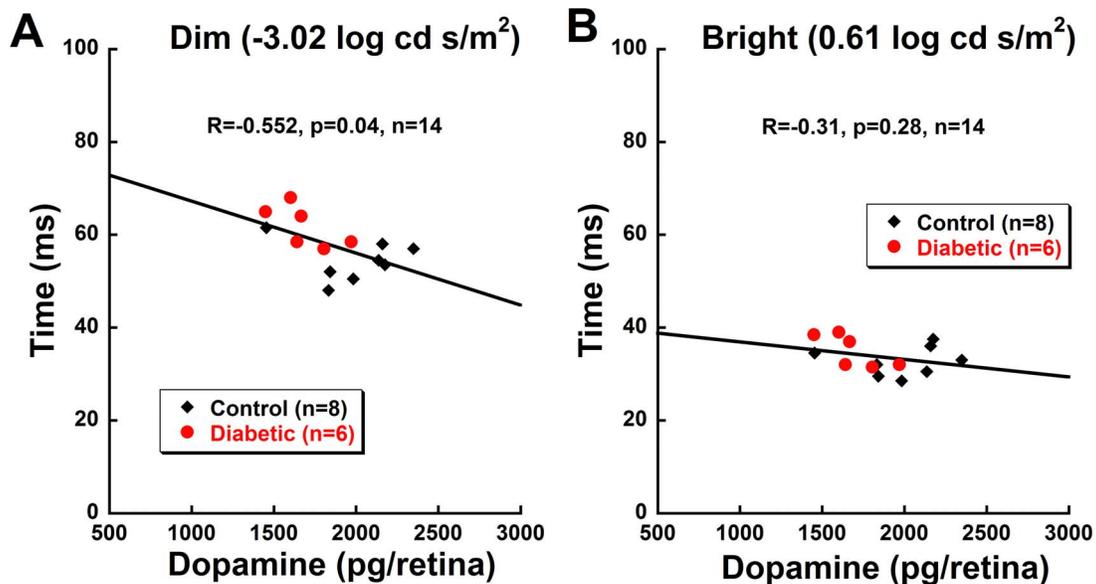
identified as potential predictive markers for the progression of neuronal dysfunction in early diabetes, OP delays were the only ERG component that showed deficits at the time points studied here.

While the exact identity of OP generators remains unclear, pharmacologic studies have shown that selective blockage of dopaminergic amacrine cells can attenuate the activity of the first OP,<sup>53</sup> and inhibition of second- and third-order interneurons of the depolarizing ON-pathway decreases the amplitudes of intermediate and late-appearing OPs.<sup>48</sup> Since we observed similar delays in all OP wavelets, it would appear the inner retinal neurons generating all four OP peaks are affected by the diabetic insult. However, it should be noted that OP3 and OP4 showed larger than normal amplitudes in the current dataset, which may indicate alterations in inhibitory signals to the neurons generating these wavelets. Supporting the possibility that chronic hyperglycemia insults inner retinal neurons directly, elevated glucose induced changes in AMPA receptor subunit distribution and localization in cultured retinal neural cells.<sup>54</sup> In addition, AII amacrine cells in diabetic rats after 3 weeks of STZ had reduced Ca(2<sup>+</sup>) permeability of extrasynaptic AMPA receptors.<sup>55</sup>

Systemic hyperglycemia has been reported to decrease amplitude and/or delay implicit time in second-order inner retinal neurons (bipolar cells, amacrine cells, ganglion cells) and the photoreceptors.<sup>24</sup> However, in the present study at an early stage of diabetes, we did not detect abnormal electrical activities of the photoreceptors as measured by a-wave amplitudes (Figs. 1A, 1B) or implicit times (data not shown), in agreement with our previous results.<sup>33,35</sup> While changes in photoreceptor structure and function have been reported that fit within the time course of the current experiment,<sup>56–58</sup> others have demonstrated unaltered a-wave responses during the course of 6 to 12 weeks of hyperglycemia.<sup>25,27,28,30,32</sup> Evidence is accumulating that diabetes elicits photoreceptor dysfunction at some point during the progression of retinopathy,<sup>56,59–63</sup> but it may not be at a detectable level within the 5-week period tested in the current study.

### Hyperglycemia Induces Dopamine-Mediated Neuronal Dysfunction

We found an association of hyperglycemia and the disruption of DA-mediated inner retinal function by employing rTHKO mice and L-DOPA treatments. Loss of retinal DA may explain



**FIGURE 6.** Dopamine levels are highly correlated with delays in inner retinal function in response to rod-driven stimuli. Scatter plot of OP2 implicit time at dim ( $-3.02 \log \text{cd s/m}^2$ ; A) and bright ( $0.61 \log \text{cd s/m}^2$ ; B) flash stimuli and corresponding retinal dopamine levels from the same mice at 5 weeks post STZ. Pearson correlation was performed on the total population ( $n = 14$ ). Diabetic animals are shown with *solid red circles*; controls are shown with *solid black diamonds*. (A) There is a significant negative correlation ( $R = -0.552$ ,  $P = 0.04$ ) between the implicit time of OP2 and the retinal dopamine level in response to dim flash stimulus. Similar correlations for OP1, OP3, and OP4 delays and dopamine levels were found with the dim flash. In contrast, with bright flash stimulus (B), no correlations ( $P = 0.28$ ) were observed between the two variables. OP1, OP3, and OP4 also did not show correlations with bright flash stimulus.

the early inner retinal dysfunction in diabetes, as drastically lowering *Tb* in the diabetic retina resulted in larger OP delays than diabetes alone and as L-DOPA was able to ameliorate the OP deficits in diabetic mice. Data reported here are in agreement with our previous findings of the therapeutic effect of L-DOPA on visual signals in early-stage diabetes.<sup>35</sup> Moreover, the severity of retinal DA deficiency caused by diabetes may reflect the degree of inner retinal dysfunction, as suggested by our findings of worsening OP delays when retinal DA levels were drastically diminished in rTHKO mice and our correlational results in Figure 6.

Underlining DA's integral role in retinal function, we previously showed that diabetic rTHKO mice develop functional deficits beyond OP delays, including b-wave delays and optokinetic tracking impairments.<sup>35</sup> Additionally, diabetic mice with normal retinal *Tb* genes have shown a decrease in retinal DA at the early stage of 4 weeks post STZ, though *Tb* transcript levels were unaltered, suggesting that the mechanisms which lead to eventual loss of DA synthesis may be multifaceted and disease-duration dependent.<sup>35</sup>

In addition, we found that most of the retinal functional deficits were observed in response to dim stimuli, suggesting that in early-stage diabetes, mainly rod-driven pathways are affected. Other studies have also reported increased susceptibility of rod pathways to hyperglycemia insult.<sup>8,33,64,65</sup> However, only with more severe loss of DA in the rTHKO mice results in significantly delayed OPs in response to both dim and bright flash stimuli. This suggests that cells or circuits involved in cone-mediated signaling are less susceptible to DA depletion compared to rod-mediated signaling cells. Supporting the sensitivity of rod-driven pathways to DA, function was significantly improved with L-DOPA in rTHKO mice in response to dim flashes only.

While we have focused on the role of DA in altering inner retinal function in diabetes, we cannot rule out the involvement of GABAergic amacrine cells and the co-release of  $\gamma$ -aminobutyric acid (GABA; inhibitory) from the dopaminergic

amacrine cells.<sup>66</sup> GABA metabolism is altered in STZ-induced rats and partially credited to the reduction of OP amplitudes and delay in their implicit times as early as 6 weeks post STZ.<sup>67</sup> Furthermore, STZ-induced diabetic mice have early deficits in inhibition in the rod pathway.<sup>65</sup>

## CONCLUSIONS

The present study contributes to our understanding of one of the underlying mechanisms for altered signal transmission by inner retinal neurons in the presence of hyperglycemia. We have demonstrated that (1) early inner retinal dysfunction in diabetes can be partly attributed to DA deficiency; (2) restoration of DA levels using L-DOPA can ameliorate inner retinal deficits in early-stage diabetes; and (3) dim, rod-dominated flash stimuli have greater sensitivity for showing OP delays than bright, mixed rod-cone flashes, implicating increased susceptibility of rod-driven pathways to hyperglycemic insult. These findings suggest that rod-driven OP delays may provide a detection method for early-stage DR, hence revealing a potentially earlier treatment window, and that the retinal dopaminergic system could be a novel therapeutic target for early-stage diabetic retinopathy. Additional studies are needed to determine if slowing the early neuronal changes will prevent or slow the late-stage vision loss in DR.

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