Rod phototransduction and light signal transmission during type 2 diabetes

Running Title: Outer Retina Function in Diabetes

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Abstract

Diabetic retinopathy is a major complication of diabetes recently associated with attenuated photoreceptor function. Multiple stressors in diabetes, such as hyperglycemia, oxidative stress and inflammatory factors, have been identified, but systemic effects of diabetes on outer retina function are incompletely understood. *Ex vivo* ERG presents the unique opportunity to test rod photoreceptor light signal transduction and transmission and determine whether they are permanently compromised and/or adapt to hyperglycemia in chronic type 2 diabetes. Light-evoked rod photoreceptor signals in control and diabetic mice *in vivo* were compared to those recorded *ex vivo* under normal or elevated extracellular glucose. Transduction and transmission of light signals were compromised in 6 mo. diabetic mice *in vivo*. In contrast, *ex vivo* rod signaling was similar in isolated retinas from 6 mo. control and diabetic mice under normoglycemic conditions. Acutely elevated glucose *ex vivo* increased light-evoked photoreceptor responses in control mice, but did not affect light responses in diabetic mice. In summary, our data suggest that long-term diabetes does not irreversibly change the ability of rod photoreceptors to transduce and mediate light signals. However, type 2 diabetes appears to induce adaptational changes in the rods that render them less sensitive to increased availability of glucose.

Diabetic retinopathy, one of the leading causes of irreversible vision loss worldwide (1), has traditionally been characterized by retinal vascular abnormalities. Recent studies in human patients with diabetes suggest, however, that retinal diabetic neuropathy with neuroglial dysfunction and degeneration frequently precedes visible microvasculopathy (2-4). Even in the absence of or with minimal signs of retinal vascular abnormalities, patients with diabetes show dysfunction and loss of retinal neuronal cells. In fact, impairment of the neurovascular unit has been suggested to lead to or accelerate retinal vascular disease progression, since implicit time delay of the multifocal electroretinogram (ERG) predicts onset of visible vascular changes (5). Damage to nerve fiber, ganglion cell and inner plexiform layers in the human eye has been demonstrated in diabetes by impaired pattern ERG and oscillatory potentials (6) and thinning of inner retinal layers (4). Reduced amplitudes and longer implicit times of the scotopic and photopic b- waves (6) also implicate dysfunction of rod and cone bipolar cells in retinal diabetic neuropathy of human patients. These findings are not universal, however, and reduced scotopic a- and b-wave amplitudes have not been shown in all studies (7).

Murine models of diabetes share many features of retinal dysfunction and cell loss with those in patients with diabetes. The positive component of the scotopic threshold response, as a measure of retinal ganglion cell function, is attenuated (8) and nerve fiber and ganglion cell layer thickness and retinal ganglion cell density are reduced (4) in streptozotocin (STZ)-induced diabetes in the rat and mouse, a type 1 diabetes model, although this is not universal in all mouse strains (9). Loss of amacrine cells observed in type 1 diabetes models (10; 11) has been suggested to underlie reduced amplitude and delayed implicit time of oscillatory potentials (8). Similarly to human studies, outer retinal dysfunction and cell loss in diabetic animal models remain controversial. Rod photoreceptor and bipolar cell dysfunction, based on reduced

amplitudes and delayed implicit times of the scotopic a- and b-waves, were reported in the db/db mouse, a type 2 diabetes model (12). Photoreceptor death was also reported in a rat STZ model (13), however, other studies did not observe loss of photoreceptors (14; 15) and reported no or only minor changes in the scotopic ERG (8), or even increased photoreceptor function in Zucker Diabetic Fatty (ZDF) rats, a type 2 diabetes model (16). Pigment regeneration and dark adaptation have not been extensively studied in diabetic animal models or human patients, but some studies have suggested deficits in pigment regeneration or dark adaptation caused by diabetes (17; 18).

Functional discrepancies in previous human and animal studies remain unexplained. It is possible that outer retinal cells are not permanently damaged, but that their function is modulated by systemic factors. Transient hyperglycemia in patients with diabetes was associated with increased scotopic b- and photopic a- and b-wave amplitudes (19). While the multifocal ERG waveform was delayed in normoglycemic patients with diabetes compared to healthy volunteers, this was partly reversed by elevation of blood glucose to their usual level of (habitual) hyperglycemia (20; 21). These findings indicate that both acute hyperglycemia and chronic diabetes modulate retinal signaling. We provide evidence for our hypothesis that long-term uncontrolled type 2 diabetes in db/db mice does not permanently alter intrinsic mechanisms in rod photoreceptors responsible for transducing and transmitting light signals. However, we find that rod photoreceptors in the *ex vivo* isolated diabetic retina are less sensitive to acute elevation of extracellular glucose concentration. We suggest that attenuation of the *in vivo* ERG in diabetic mice is likely due to diabetes-induced systemic factors influencing rod photoreceptors, which once removed, can no longer mediate suppression of photoreceptor responses in the context of the *ex vivo* testing.

RESEARCH DESIGN AND METHODS

Animals

Male and female C57B/6J mice and BKS.Cg-Dock7^{m} +/+ Lepr^{db}/J mice were bred in house or purchased at 8-10 weeks of age from Jackson Laboratory. Animals homozygous for the Lepr^{db} mutation (db/db) are diabetic. Age-matched heterozygotes (db/+) were used as non-diabetic controls.

Weight, blood glucose and HbA1c

Animals were kept under a 12:12 hour light/dark cycle and given ad libitum access to food and water. Diabetic status was confirmed by measuring weight, blood glucose and HbA1c at 3 or 6 months of age (Supplemental Figure 1).

Changes in blood glucose concentrations during isoflurane anesthesia were measured in C57B/6J mice immediately before anesthesia and 20 min after beginning inhalation of 2% isoflurane.

In vivo ERG

Mice were fully dark adapted and anesthetized with 1.8-3.0% isoflurane in room air using a SomnoSuite Low Flow Anesthesia System (Kent Scientific). Pupils were dilated with 2% atropine sulfate eye drops (Akorn, Illinois) and corneas lubricated with a mixture of phosphate buffered saline (PBS) with Goniovisc (2.5% hypromellose solution, Contacare, Gujarat, India). Body temperature was maintained by placement on a heated table throughout the experiment. ERG waveforms were recorded using corneal contact lens electrodes and subdermal needle

electrodes between the eyes as reference and at the base of the tail as ground electrodes. Responses to light flashes (rod stimuli, to activate rod phototransduction in rodents), were collected using a ColorDome and Espion V6 software (Diagnosys LLC).

For scotopic ERGs 20 sweeps with inter-sweep intervals of 2 s were recorded at light flash energies (Luminous energy, Q_V) from 10^{-5} to 10^{-3} cd.s.m^{-2}, 10 sweeps with 5 s intervals at 10^{-2} cd.s.m^{-2}, 5 sweeps with 10 s intervals from 10^{-1} to 0.5 cd.s.m^{-2}, 3 sweeps with 20 s intervals from 1 to 5 cd.s.m^{-2}, and 2 sweeps with a 60 s interval at 10 and 30 cd.s.m^{-2}. Oscillatory potentials were high-pass filtered above 25 Hz and subtracted from ERG waveforms. a- and b-wave response amplitudes were measured between baseline and trough and between trough and peak, respectively and were plotted as the response amplitude over the log light intensity. a- and b-wave implicit times were determined as time from stimulus onset to peak. The amplification constant *A* of phototransduction at 1 cd.s.m^{-2} was calculated as described by Lamb & Pugh (22).

To assess dark adaption, rod b-wave sensitivity (S_f = b-wave amplitude/Q_V) was initially determined in fully dark-adapted animals averaging 10 light flashes with 10 s inter-flash intervals at 0.25 cd.s.m^{-2} (S_{fDA}). Then, bright bleaching light illumination of 1000 cd.m^{-2} was applied for 30 s, which is predicted to bleach ~90% of rod photopigments. Light stimuli with decreasing Q_V ranging from 2 sweeps with a 30 s interval at 50.25 cd.s.m^{-2} to 10 sweeps with 10 s intervals at 0.25 cd.s.m^{-2} were applied after the bleach to assess recovery of b-wave S_f over 59 min. Sensitivity data are displayed as the S_f/S_{fDA} ratio over time.

Ex vivo ERG

Mice were fully dark adapted overnight and sacrificed by CO_2 asphyxia. Eyes were enucleated and dissected in Ames' media supplemented with 0.25 mM sodium glutamate (23) and

oxygenated with 95% O_2/5% CO_2. The anterior part of the eye and the lens were removed and retinas were carefully dissected and mounted on an *ex vivo* specimen holder, as previously described (24). Tissue was continuously superfused with oxygenated Ames' media containing 0.25 mM sodium glutamate heated to 36□C. Dissected eye cups from fellow eyes were stored in oxygenated Ames' media at room temperature before used for isolated retina recordings within 2 hours. ERG waveforms were elicited by light stimuli of increasing intensity at a wavelength of 530 nm, amplified using a DP-311 differential amplifier (Warner Systems), digitized with a MEA2100-System (Multichannel Systems), and recorded using Multi Channel Experimenter software. To investigate acute effects of high glucose, we added either 30 mM D-glucose or mannitol as iso-osmotic control to the perfusate in fellow eyes 20 min before recordings were started.

To determine PII responses (originating from ON bipolar cells), combined fast PIII (photoreceptor component) and PII (bipolar cell component) responses were measured at light intensities ranging from 3 to 20 photons μm^{-2} in the presence of 100 μM $BaCl_2$ (to inhibit K^+ channels in Müller glia) (24). Corresponding fast PIII responses were recorded in the presence of 100 μM $BaCl_2$ and 40μM DL-AP4 (to block neurotransmission from photoreceptors to ON-bipolar cells) and subtracted from combined fast PIII and PII responses to obtain PII responses (25) (Figure 2A-C). Fast PIII response families were recorded at light intensities from 30 to 1,000 photons μm^{-2} in the presence of 100 μM $BaCl_2$ and 40 μM DL-AP4. The amplification constant *A* for phototransduction at a light intensity of 50 photons μm^{-2} was calculated, as previously described (22).

Statistical analysis

Data are displayed as arithmetic means ± standard error of the mean. Statistical analysis was performed using Origin software. Differences between two groups with independent values were determined by two-tailed student's t-test, for multiple stimulus intensities by two-way ANOVA.

Data and Resource Availability

No datasets and no applicable resources were generated or analyzed during the current study.

RESULTS

Type 2 diabetes has different effects on outer retinal physiology under *in vivo* and *ex vivo* conditions

Long-term diabetes has been shown to impair the function and survival of retinal ganglion and amacrine cells (4; 6; 8; 10; 11), but there is currently no consensus on its effects on phototransduction and synaptic transmission of the rod photoreceptors. Here we first asked: what is the time course of alterations in rod-mediated light signals caused by type 2 diabetes in habitual blood glucose? To answer this question, we compared scotopic *in vivo* ERG a- and b-waves in db/db mice and their age-matched db/+ controls at 3 and 6 months of age. We used isoflurane anesthesia, which in contrast to ketamine/xylazine anesthesia, did not significantly increase blood glucose in C57BL/6J mice (Supplemental Figure 1C, (26; 27)).

We did not observe reduced *in vivo* a- and b-wave amplitudes in diabetic compared to non-diabetic control mice at 3 months of age, but rather a slight increase of the *in vivo* a-wave amplitude, similar to that reported by Johnson *et al.* (16) (Figure 1A-C). a- and b-wave implicit

times were delayed at this age, suggesting beginning photoreceptor and ON bipolar cell dysfunction (Figure 1G-H). Conversely, in 6 month old diabetic mice the scotopic *in vivo* ERG a- and b-wave amplitudes were reduced (Figure 1D-F) and a- and b-wave implicit times were delayed (Figure 1I-J). The amplification constant (*A*) of phototransduction, a measure of signal amplification in photoreceptors, was determined based on the leading edge of the a-wave, when b-wave intrusion is negligible, as previously described (28). *A* was not significantly different between 6 month old control and diabetic mice (n=8-9, p=0.25), indicating that the activation reactions of phototransduction in rods are not affected by diabetes. Interestingly, we found a trend towards reduced b/a-wave ratio in diabetic animals compared to non-diabetic controls at 3 months of age, which was highly significant at 6 months of age (Table 1).

Although *in vivo* ERG data by us and others suggest that the light-sensitive current and/or synaptic transmission of rods are compromised during long-term hyperglycemia, it is not known whether these changes are related to an altered extracellular environment or permanent molecular/structural changes in rods or rod bipolar cells caused by diabetes. Moreover, systemic effects of anesthesia, such as hyperglycemia, complicate interpretation of the results obtained by *in vivo* ERG in animal models of diabetes. To determine the effects of chronic diabetes on the intrinsic functional properties of rods and rod bipolar cells in a normoglycemic environment and to avoid systemic complications of anesthesia, we assessed rod phototransduction and synaptic transmission using *ex vivo* ERG in 6 month old control and diabetic mice. This technique also allowed us to pharmacologically isolate and quantify the fast PIII (rod photoreceptor) and PII (ON bipolar cell) components of the ERG signal (Figure 2A-C). Surprisingly, in contrast to the *in vivo* ERG, the amplitudes of the fast PIII or PII components were not significantly changed in 6 month old db/db mice as compared to age-matched db/+ mice (Figure 2D-E). Contrary to *in*

vivo ERG findings, the *ex vivo* implicit times of the fast PIII component shortened, with no change seen in the PII time to peak caused by diabetes (Figure 2H-I). These results demonstrate that transduction or transmission of light signals in rods is not adversely affected by long-term type 2 diabetes under the standard physiological environment used in *ex vivo* experiments.

Although our amplitude analysis of the *ex vivo* ERG data suggests that diabetes does not alter the light sensitivity of rod photoreceptors in normal glucose, it is possible that the kinetics of phototransduction activation and inactivation reactions are modulated by diabetes. To test this, we determined the gain of the phototransduction activation reactions (*A*) and time-to-peak (=implicit time) from *ex vivo* ERG fast PIII (photoreceptor) responses to dim flashes (Figure 2F-G). Interestingly, we found that long-term type 2 diabetes increased *A* and shortened the time-to-peak, suggesting an overall acceleration of both phototransduction activation and inactivation reactions.

Chronic type 2 diabetes does not compromise photoreceptor dark adaptation under habitual blood glucose

Slow dark adaptation of the parafoveal rods is an early symptom in age-related macular degeneration (29). However, whether dark adaptation or pigment regeneration of rods is altered in diabetes is less clear, with few studies having shown alterations in animal models or patients with diabetes (17; 18). To determine whether rod photoreceptor dark adaptation and pigment regeneration are affected by long-term type 2 diabetes in habitual blood glucose levels, we compared how the sensitivity of *in vivo* ERG b-wave responses recovered following a substantial bleach of the visual pigments. In contrast to some previous studies (18; 30), we did not observe any difference in the dark adaptation kinetics or capacity between 6 months old diabetic and non-

diabetic animals (Figure 3), indicating that db/db mice with habitual high blood glucose concentrations can dark adapt normally.

Acute hyperglycemia promotes light responses in rod photoreceptors of non-diabetic, but not diabetic (type 2) mouse retinas

Previous clinical studies demonstrating increased b-wave amplitude following elevation of blood glucose suggest that retinal function may be modulated by extracellular glucose concentration (19). To investigate this, we used the *ex vivo* ERG method that allows us to precisely control glucose concentrations. We first evaluated whether acute elevation of extracellular glucose in the perfusate altered light evoked activity of the rod photoreceptors or bipolar cells in C57BL6/J retinas. Supplementation with 30 mM glucose compared to mannitol iso-osmotic control increased the maximal photoreceptor response (fast PIII) amplitude (Figure 4A, C). The bipolar cell (PII) responses were also slightly increased by acute addition of glucose (Figure 4B, D). However, the PII/fast PIII ratio was not significantly changed between mannitol and glucose supplementation, indicating that the increased PII amplitude in the presence of added glucose derives from the increased photoreceptor responses.

Next, we wanted to know if long-term diabetes alters the acute hyperglycemia-induced augmentation of photoreceptor responses. Diabetes has been shown to cause various long-term adaptations in the retina, including differential expression of glucose transporters (31). Thus, we hypothesized that the photoreceptors in 6 months old db/db mice are less sensitive to elevated extracellular glucose. Indeed, fast PIII amplitudes of db/+ control retinas were significantly augmented in acute hyperglycemia, whereas the photoreceptor responses of db/db retinas were not affected by high glucose exposure (Figure 5A-C). On the other hand, we did not observe a significant modulation of the PII amplitudes by elevated glucose exposure in control or diabetic

mice (Figure 5D-F), resulting in a reduced PII/fast PIII ratio (data not shown, *$p<0.05$, n=4-5), indicative of disrupted signal transmission from photoreceptors to ON-bipolar cells caused by acute hyperglycemia. We observed delayed PIII implicit times in high glucose compared to mannitol iso-osmotic control in both db/+ and db/db retinas (Figure 5G-I). While the fast PIII implicit times in db/db retinas were accelerated compared to db/+ retinas in mannitol iso-osmotic control (Figure 2H), no significant difference was observed between non-diabetic retinas in normal glucose (with 30 mM mannitol) and diabetic retinas in high glucose (Figure 5G-H). The amplification constant *A* as described above was not different between high glucose and mannitol treatment in either db/+ or db/db mice (data not shown), suggesting that the increased implicit times of the rod responses must be due to decelerated phototransduction deactivation rather than activation reactions caused by the elevated glucose. Similar to the PIII times to peak, the PII implicit times lengthened by supplementation with glucose compared to mannitol in both non-diabetic and diabetic animals (Figure 5I-J). These results indicate that light-evoked potentials of rod photoreceptors from normoglycemic mice can be promoted by acute high glucose treatment. However, long-term diabetes desensitizes photoreceptors to acute hyperglycemia and allows them to maintain normal (lower) light-sensitive current under hyperglycemic environment.

DISCUSSION

It is widely accepted that diabetes results in loss of retinal ganglion and amacrine cells and associated attenuation of oscillatory potentials, both in diabetic animal models and in patients (4;

8; 10; 32). Effects of diabetes on photoreceptor and ON-bipolar cell light signaling remain controversial, however. While many studies demonstrated attenuated a- and b-wave amplitudes of the scotopic *in vivo* ERG (11; 12; 30; 33), other reports are contradictory (8; 16; 34). Causes of these discrepancies remain largely unclear, but have previously been ascribed to duration of diabetes (11; 33). This is supported by our study, which shows reduction of *in vivo* a- and b-wave amplitudes in db/db mice at 6 months of age, whereas the scotopic a-wave was increased in 3 months old diabetic mice (Figure 1 D-F and 1 A-C, respectively). Based on our data, we conclude that changes in *in vivo* rod photoreceptor function are minor at 3 months of age, but significant dysfunction occurs in uncontrolled diabetes (type 2) at 6 months of age. Our data also indicate that signal transmission from rods to rod bipolar cells, measured by the b/a-wave ratio, is impaired in db/db mice, which progresses from 3 to 6 months of age.

Importantly, though, our data imply that altered photoreceptor function *in vivo* (Figure 1) may not be explained by intrinsic dysfunction of the light signal transduction or transmission in diabetic rods (Figure 2), but by systemic diabetes-mediated changes that alter rod function. This is consistent with lack of photoreceptor loss (35) and absence of reports of reduced scotopic ERGs in patients with diabetes. Since other studies have shown attenuated photoreceptor function in *ex vivo* transretinal recordings from STZ mice (36), our findings may not be universal and restricted to the db/db mouse as a model of type 2 diabetes. It is also possible that compromised rod photoreceptor function in the STZ mouse model is due to known neurotoxic effects of STZ (37) rather than a consequence of diabetes. Consistent with a recent study by Calbiague *et al.*, we also did not find evidence for impaired ON bipolar cell function in diabetic retinas *ex vivo (38)*.

The *ex vivo* ERG technique uniquely allows precise control of the experimental conditions, including extracellular glucose concentration, and isolation of the ERG waveforms from individual retinal cell types. We, therefore, conclusively show that under normoglycemic conditions and in the absence of detrimental factors associated with diabetes, such as oxidative stress, generation of light signals and their transmission to rod bipolar cells are not altered by long-term diabetes. What could explain the difference between *in vivo* and *ex vivo ERG* in diabetic mice? One obvious difference is the hyperglycemic environment of photoreceptors in diabetic mice *in vivo* (39) compared to normal extracellular glucose in the *ex vivo* experiments for retinas from both control and diabetic mice. However, increasing the glucose concentration in *ex vivo* experiments with diabetic mice did not affect their photoreceptor response amplitudes (Figure 5A,C). Thus, the difference in the extracellular glucose *in vivo* cannot explain the decreased *in vivo* ERG amplitudes in our experiments.

Other confounding factors include anesthesia-induced systemic effects. Indeed, anesthetic agents are known to affect ERG amplitudes (40). Thus, one possibility is a different response of retinas in diabetic mice to the systemic changes caused by anesthesia. This is supported by the observation that scotopic a-waves are not altered in conscious human patients with diabetes during *in vivo* ERG experiments (41). One obvious systemic effect of anesthesia is elevated blood glucose (27). However, previous studies have demonstrated that hyperglycemia in fact promotes retinal signaling in diabetic patients (19; 20), while in this study light-evoked *ex vivo* responses from diabetic mouse retinas were not changed by hyperglycemia. Thus, it is improbable that anesthesia-induced hyperglycemia could explain reduced a- or b-waves in the *in vivo* ERG experiments from 6 month old db/db mice. Furthermore, we used isoflurane anesthesia which did not cause a significant anesthesia-induced hyperglycemia. While previous studies

concluded that retinal neurodegeneration in db/db mice was abrogated by dietary restriction, it remains unclear whether this was the result of reduced hyperglycemia, body weight or other diabetes-related changes (12). Consequently, we suggest that systemic effects, other than hyperglycemia, that are either related to diabetes or general anesthesia affect photoreceptor light responses, resulting in reduced a- and b-wave amplitudes *in vivo* after long-term uncontrolled diabetes in mice.

It is also possible that altered recording geometry due to different size of the diabetic animals or their altered body lipid composition affects the *in vivo* ERG amplitudes. This, however, is not a probable explanation since altered *in vivo* ERG a- and b-waves have been reported both in type 1 (non-obese) and 2 (obese) diabetic animal models. Overall, we conclude that photoreceptor and bipolar cells in the retinas from diabetic mice have the potential to mediate normal light signals that may have been negatively impacted by altered extracellular environment caused by diabetes *in vivo*.

While photoreceptors are highly metabolically active and mostly depend on glucose as their source of energy (42), effects of acute hyperglycemia on retinal function remain unclear. Studies in patients with diabetes and diabetic animal models suggest a correlation between bipolar cell function and blood glucose levels. In clinical studies the scotopic b-wave amplitude was increased in hyperglycemic diabetes patients (19). Similarly, the scotopic a- and b-waves were reported to be increased in diabetic ZDF rats, while acute insulin treatment resulted in decreased a-wave amplitudes (16). These results seem at odds with our observation that acutely elevated glucose increased photoreceptor responses in control but not in 6 months old db/db mice. However, in human patients glucose levels are typically controlled by insulin suggesting that the ability to promote photoreceptor responses is lost specifically due to a long-term exposure to an

uncontrolled hyperglycemic environment. The difference of our data in comparison to that of Johnson *et al.* remain unclear, but may be related to different animal models and longer duration of diabetes in our study.

While our experiments did not identify the mechanisms by which light-evoked photoreceptor responses are increased in the presence of additional glucose, likely targets include energy dependent processes in photoreceptors such as Na^+/K^+ ATPases, $Na^+/Ca^{2+}/K^+$ exchangers and Ca^{2+} ATPases (42), ATP-sensitive K^+ channels expressed in photoreceptors (43) or other ATP-dependent processes. The main glucose transporters expressed in photoreceptors are the insulin-independent GLUT-1 and insulin-dependent GLUT-4 (42), which have previously been reported to be reduced in diabetes (31; 44; 45), thus potentially explaining the inability of the photoreceptors in db/db mice to utilize increased glucose concentrations to promote their light responses. However, these findings are not universal and increased expression of GLUT-1 in diabetes has also been reported (39). Determining underlying causes for the lack of increased photoreceptor function during acute hyperglycemia in diabetic animals was beyond the scope of this study and should be subject to future investigations.

Effects of long-term diabetes on dark adaptation remain unclear, with subtle deficits reported in diabetic patients (18; 46). Data from diabetic animal models remains controversial, with a recent study showing deficits in rhodopsin regeneration (17) and another demonstrating faster recovery of rod sensitivity after 20% bleach in diabetic rats (30). We demonstrate no change in the recovery kinetics of the rod b-wave sensitivity following ~90% bleach of rhodopsin in 6 month old db/db mice as compared to their age-matched db/+ controls under habitual blood glucose. This is consistent with a lack of reports of gross dark adaptation deficits in diabetic patients, specifically in comparison to the well documented delayed dark adaptation of the parafoveal rods

in AMD patients (47). Indeed, it has been shown that dark adaptation of hyperglycemic diabetic patients is normal, but slowed if blood glucose is lowered to normoglycemic levels (18), which is in agreement with our observation that hyperglycemic db/db mice dark adapt normally.

In summary, we demonstrate that the rods have potential to transduce and transmit light signals normally in db/db mice at least up to 6 month of age under standard *ex vivo* environment. Differences between diabetic and non-diabetic mice in the *in vivo* ERG are likely due to systemic factors, which remain to be identified.

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References

1. Resnikoff S, Pascolini D, Etya'ale D, Kocur I, Pararajasegaram R, Pokharel GP, Mariotti SP: Global data on visual impairment in the year 2002. Bull World Health Organ 2004;82:844-851
2. Antonetti DA, Barber AJ, Bronson SK, Freeman WM, Gardner TW, Jefferson LS, Kester M, Kimball SR, Krady JK, LaNoue KF, Norbury CC, Quinn PG, Sandirasegarane L, Simpson IA, Group JDRC: Diabetic retinopathy: seeing beyond glucose-induced microvascular disease. Diabetes 2006;55:2401-2411
3. Rajagopal R, Bligard GW, Zhang S, Yin L, Lukasiewicz P, Semenkovich CF: Functional Deficits Precede Structural Lesions in Mice With High-Fat Diet-Induced Diabetic Retinopathy. Diabetes 2016;65:1072-1084
4. Sohn EH, van Dijk HW, Jiao C, Kok PH, Jeong W, Demirkaya N, Garmager A, Wit F, Kucukevcilioglu M, van Velthoven ME, DeVries JH, Mullins RF, Kuehn MH, Schlingemann RO, Sonka M, Verbraak FD, Abràmoff MD: Retinal neurodegeneration may precede microvascular changes characteristic of diabetic retinopathy in diabetes mellitus. Proc Natl Acad Sci U S A 2016;113:E2655-2664
5. Ng JS, Bearse MA, Schneck ME, Barez S, Adams AJ: Local diabetic retinopathy prediction by multifocal ERG delays over 3 years. Invest Ophthalmol Vis Sci 2008;49:1622-1628
6. Lecleire-Collet A, Audo I, Aout M, Girmens JF, Sofroni R, Erginay A, Le Gargasson JF, Mohand-Saïd S, Meas T, Guillausseau PJ, Vicaut E, Paques M, Massin P: Evaluation of retinal function and flicker light-induced retinal vascular response in normotensive patients with diabetes without retinopathy. Invest Ophthalmol Vis Sci 2011;52:2861-2867
7. Holopigian K, Greenstein VC, Seiple W, Hood DC, Carr RE: Evidence for photoreceptor changes in patients with diabetic retinopathy. Invest Ophthalmol Vis Sci 1997;38:2355-2365
8. Kohzaki K, Vingrys AJ, Bui BV: Early inner retinal dysfunction in streptozotocin-induced diabetic rats. Invest Ophthalmol Vis Sci 2008;49:3595-3604
9. Kern TS, Barber AJ: Retinal ganglion cells in diabetes. J Physiol 2008;586:4401-4408
10. Gastinger MJ, Singh RS, Barber AJ: Loss of cholinergic and dopaminergic amacrine cells in streptozotocin-diabetic rat and Ins2Akita-diabetic mouse retinas. Invest Ophthalmol Vis Sci 2006;47:3143-3150
11. Hombrebueno JR, Chen M, Penalva RG, Xu H: Loss of synaptic connectivity, particularly in second order neurons is a key feature of diabetic retinal neuropathy in the Ins2Akita mouse. PLoS One 2014;9:e97970

12. Bogdanov P, Corraliza L, Villena JA, Carvalho AR, Garcia-Arumí J, Ramos D, Ruberte J, Simó R, Hernández C: The db/db mouse: a useful model for the study of diabetic retinal neurodegeneration. PLoS One 2014;9:e97302
13. Park SH, Park JW, Park SJ, Kim KY, Chung JW, Chun MH, Oh SJ: Apoptotic death of photoreceptors in the streptozotocin-induced diabetic rat retina. Diabetologia 2003;46:1260-1268
14. Liu H, Tang J, Du Y, Saadane A, Tonade D, Samuels I, Veenstra A, Palczewski K, Kern TS: Photoreceptor Cells Influence Retinal Vascular Degeneration in Mouse Models of Retinal Degeneration and Diabetes. Invest Ophthalmol Vis Sci 2016;57:4272-4281
15. Du Y, Veenstra A, Palczewski K, Kern TS: Photoreceptor cells are major contributors to diabetes-induced oxidative stress and local inflammation in the retina. Proc Natl Acad Sci U S A 2013;110:16586-16591
16. Johnson LE, Larsen M, Perez MT: Retinal adaptation to changing glycemic levels in a rat model of type 2 diabetes. PLoS One 2013;8:e55456
17. Malechka VV, Moiseyev G, Takahashi Y, Shin Y, Ma JX: Impaired Rhodopsin Generation in the Rat Model of Diabetic Retinopathy. Am J Pathol 2017;187:2222-2231
18. Holfort SK, Jackson GR, Larsen M: Dark adaptation during transient hyperglycemia in type 2 diabetes. Exp Eye Res 2010;91:710-714
19. Holfort SK, Klemp K, Kofoed PK, Sander B, Larsen M: Scotopic electrophysiology of the retina during transient hyperglycemia in type 2 diabetes. Invest Ophthalmol Vis Sci 2010;51:2790-2794
20. Klemp K, Larsen M, Sander B, Vaag A, Brockhoff PB, Lund-Andersen H: Effect of short-term hyperglycemia on multifocal electroretinogram in diabetic patients without retinopathy. Invest Ophthalmol Vis Sci 2004;45:3812-3819
21. Klemp K, Sander B, Brockhoff PB, Vaag A, Lund-Andersen H, Larsen M: The multifocal ERG in diabetic patients without retinopathy during euglycemic clamping. Invest Ophthalmol Vis Sci 2005;46:2620-2626
22. Lamb TD, Pugh EN: A quantitative account of the activation steps involved in phototransduction in amphibian photoreceptors. J Physiol 1992;449:719-758
23. Green DG, Kapousta-Bruneau NV: Electrophysiological properties of a new isolated rat retina preparation. Vision Res 1999;39:2165-2177
24. Vinberg F, Kefalov V: Simultaneous ex vivo functional testing of two retinas by in vivo electroretinogram system. J Vis Exp 2015:e52855
25. Green DG, Kapousta-Bruneau NV: A dissection of the electroretinogram from the isolated rat retina with microelectrodes and drugs. Vis Neurosci 1999;16:727-741
26. Schmitz S, Tacke S, Guth B, Henke J: Comparison of Physiological Parameters and Anaesthesia Specific Observations during Isoflurane, Ketamine-Xylazine or Medetomidine-Midazolam-Fentanyl Anaesthesia in Male Guinea Pigs. PLoS One 2016;11:e0161258
27. Brown ET, Umino Y, Loi T, Solessio E, Barlow R: Anesthesia can cause sustained hyperglycemia in C57/BL6J mice. Vis Neurosci 2005;22:615-618
28. Vinberg F, Kolesnikov AV, Kefalov VJ: Ex vivo ERG analysis of photoreceptors using an in vivo ERG system. Vision Res 2014;101:108-117
29. Haimovici R, Owens SL, Fitzke FW, Bird AC: Dark adaptation in age-related macular degeneration: relationship to the fellow eye. Graefes Arch Clin Exp Ophthalmol 2002;240:90-95
30. Phipps JA, Yee P, Fletcher EL, Vingrys AJ: Rod photoreceptor dysfunction in diabetes: activation, deactivation, and dark adaptation. Invest Ophthalmol Vis Sci 2006;47:3187-3194
31. Badr GA, Tang J, Ismail-Beigi F, Kern TS: Diabetes downregulates GLUT1 expression in the retina and its microvessels but not in the cerebral cortex or its microvessels. Diabetes 2000;49:1016-1021
32. Barber AJ, Lieth E, Khin SA, Antonetti DA, Buchanan AG, Gardner TW: Neural apoptosis in the retina during experimental and human diabetes. Early onset and effect of insulin. J Clin Invest 1998;102:783-791

33. Salido EM, de Zavalía N, Schreier L, De Laurentiis A, Rettori V, Chianelli M, Keller Sarmiento MI, Arias P, Rosenstein RE: Retinal changes in an experimental model of early type 2 diabetes in rats characterized by non-fasting hyperglycemia. Exp Neurol 2012;236:151-160
34. Ramsey DJ, Ripps H, Qian H: An electrophysiological study of retinal function in the diabetic female rat. Invest Ophthalmol Vis Sci 2006;47:5116-5124
35. Kern TS, Berkowitz BA: Photoreceptors in diabetic retinopathy. J Diabetes Investig 2015;6:371-380
36. Berkowitz BA, Kern TS, Bissig D, Patel P, Bhatia A, Kefalov VJ, Roberts R: Systemic Retinaldehyde Treatment Corrects Retinal Oxidative Stress, Rod Dysfunction, and Impaired Visual Performance in Diabetic Mice. Invest Ophthalmol Vis Sci 2015;56:6294-6303
37. Genrikhs EE, Stelmashook EV, Golyshev SA, Aleksandrova OP, Isaev NK: Streptozotocin causes neurotoxic effect in cultured cerebellar granule neurons. Brain Res Bull 2017;130:90-94
38. Calbiague VM, Vielma AH, Cadiz B, Paquet-Durand F, Schmachtenberg O: Physiological assessment of high glucose neurotoxicity in mouse and rat retinal explants. J Comp Neurol 2019;
39. You ZP, Zhang YL, Shi K, Shi L, Zhang YZ, Zhou Y, Wang CY: Suppression of diabetic retinopathy with GLUT1 siRNA. Sci Rep 2017;7:7437
40. Nair G, Kim M, Nagaoka T, Olson DE, Thulé PM, Pardue MT, Duong TQ: Effects of common anesthetics on eye movement and electroretinogram. Doc Ophthalmol 2011;122:163-176
41. Lovasik JV, Kergoat H: Electroretinographic results and ocular vascular perfusion in type 1 diabetes. Invest Ophthalmol Vis Sci 1993;34:1731-1743
42. Narayan DS, Chidlow G, Wood JP, Casson RJ: Glucose metabolism in mammalian photoreceptor inner and outer segments. Clin Exp Ophthalmol 2017;45:730-741
43. Ettaiche M, Heurteaux C, Blondeau N, Borsotto M, Tinel N, Lazdunski M: ATP-sensitive potassium channels (K(ATP)) in retina: a key role for delayed ischemic tolerance. Brain Res 2001;890:118-129
44. Fernandes R, Carvalho AL, Kumagai A, Seica R, Hosoya K, Terasaki T, Murta J, Pereira P, Faro C: Downregulation of retinal GLUT1 in diabetes by ubiquitinylation. Mol Vis 2004;10:618-628
45. Sánchez-Chávez G, Peña-Rangel MT, Riesgo-Escovar JR, Martínez-Martínez A, Salceda R: Insulin stimulated-glucose transporter Glut 4 is expressed in the retina. PLoS One 2012;7:e52959
46. Henson DB, North RV: Dark adaptation in diabetes mellitus. Br J Ophthalmol 1979;63:539-541
47. Owsley C, McGwin G, Clark ME, Jackson GR, Callahan MA, Kline LB, Witherspoon CD, Curcio CA: Delayed Rod-Mediated Dark Adaptation Is a Functional Biomarker for Incident Early Age-Related Macular Degeneration. Ophthalmology 2016;123:344-351

Table 1: b/a-wave ratios in diabetic mice at 3 and 6 months of age

Age	db/+	db/db	p value
3 months	2.35 ± 0.09	2.20 ± 0.10	p=0.054**
6 months	2.66 ± 0.14	2.05 ± 0.14	p<0.01

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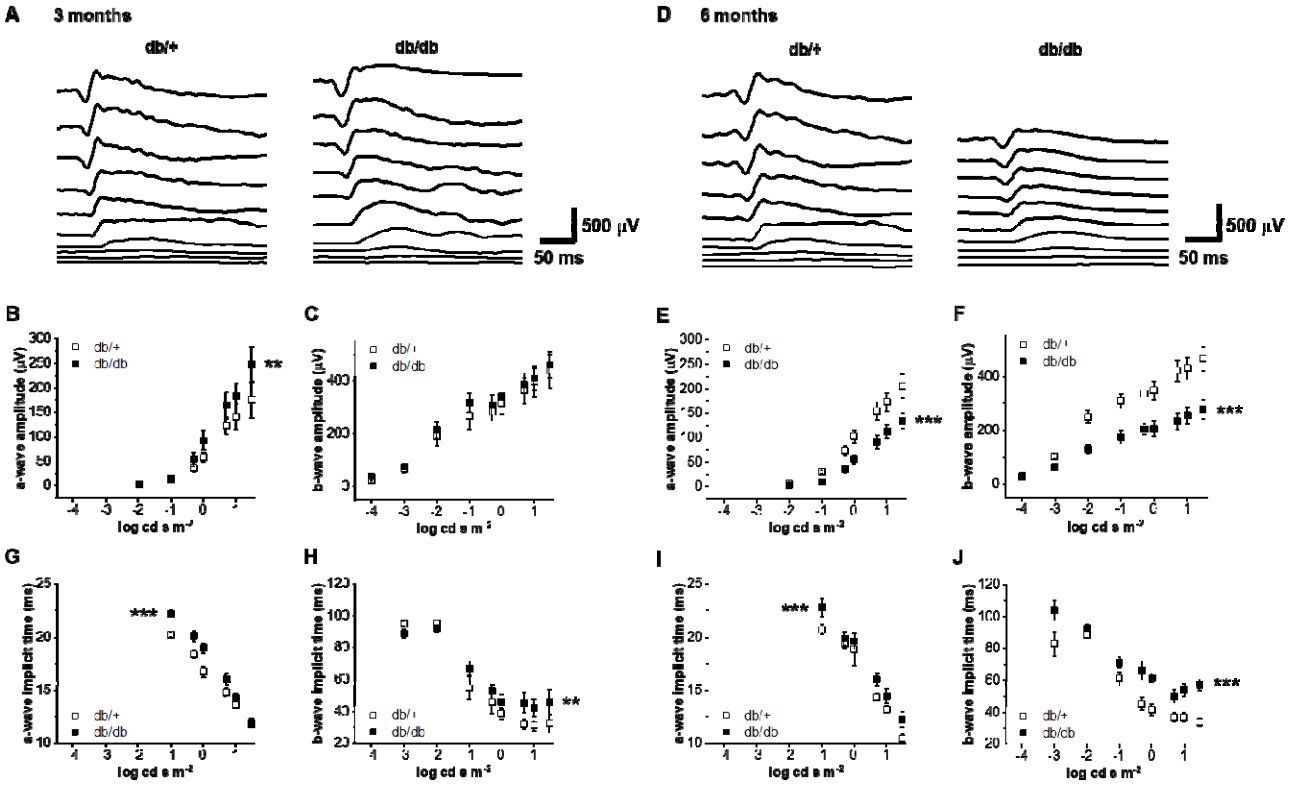


Figure 1: Scotopic a- and b-waves are reduced in 6 but not 3 months old type 2 diabetic mice *in vivo*. Representative *in vivo* ERG light responses from 3 (A) and 6 (D) month old control (db/+) and diabetic (db/db) mice to flashes of light ranging from 10^{-5} to 30 cd s m^{-2}. The scotopic a-wave (B, E) and b-wave (C, F) amplitudes as a function of flash energy (Q_V) for db/+ (white squares) and db/db (filled squares) mice at 3 (A-C, n=4-8 eyes from 2-4 animals) and 6 (D-F, n=9-10 eyes from 5 animals, *** $p<0.001$) months of age. a- and b-wave implicit times in db/db (black squares) mice compared to db/+ (white squares) mice at either time point (G-J, ** $p<0.01$, *** $p<0.001$).

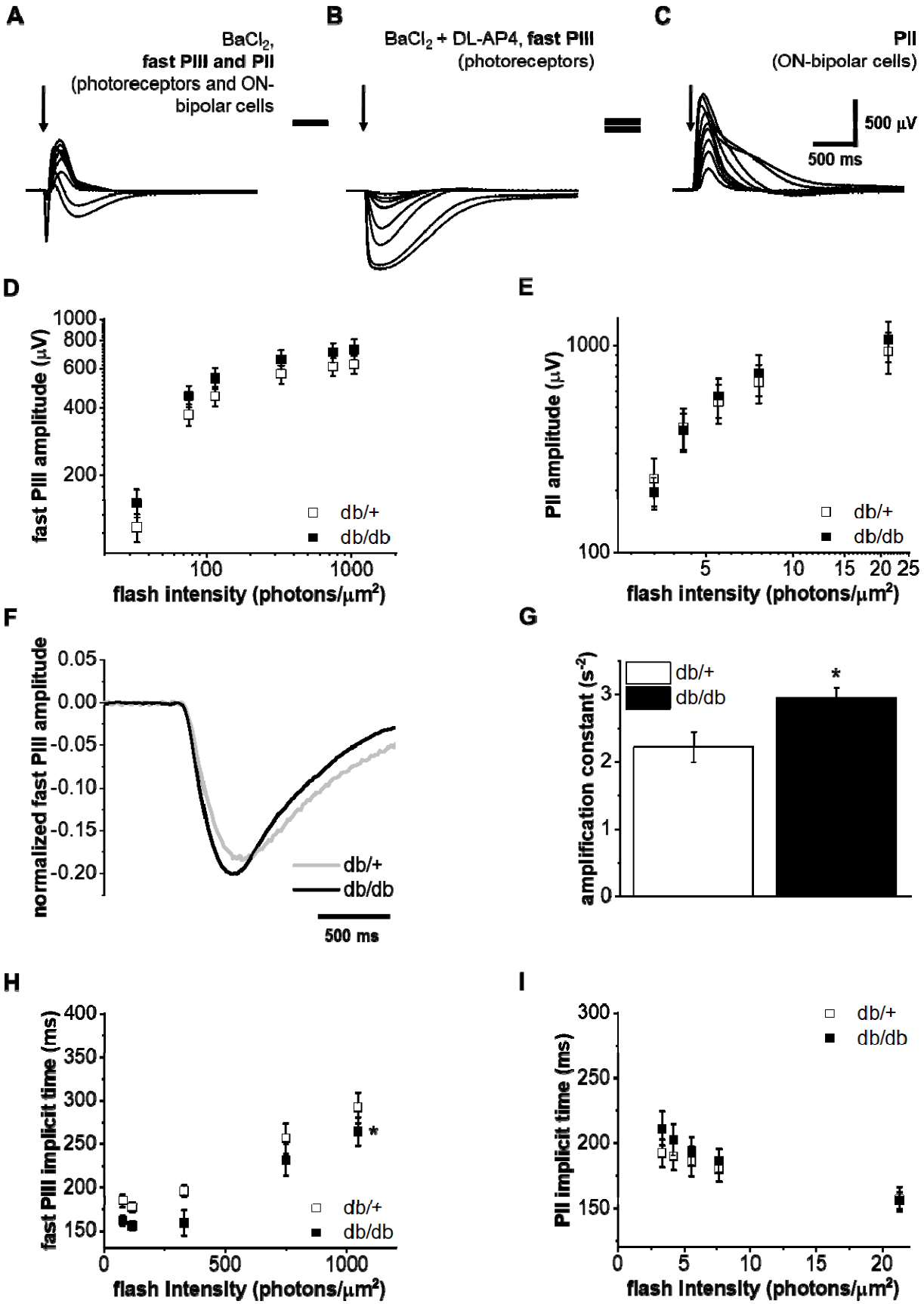

A
BaCl2,
fast PIII and PII
(photoreceptors and ON-bipolar cells
B
BaCl2 + DL-AP4, fast PIII
(photoreceptors)
C
PII
(ON-bipolar cells)
500 µV
500 ms
D
fast PIII amplitude (µV)
flash intensity (photons/µm2)
db/+
db/db
E
PII amplitude (µV)
flash intensity (photons/µm2)
db/+
db/db
F
normalized fast PIII amplitude
db/+
db/db
500 ms
G
amplification constant (s-2)
db/+
db/db
*
H
fast PIII implicit time (ms)
flash Intensity (photons/µm2)
db/+
db/db
*
I
PII implicit time (ms)
flash Intensity (photons/µm2)
db/+
db/db

Figure 2: Isolation of photoreceptor and ON-bipolar cell function by *ex vivo* ERG demonstrates no amplitude changes in the diabetic retina. The fast PIII response family as a measure of *ex vivo* photoreceptor function in this example trace was recorded in the presence of $BaCl_2$ and DL-AP4 with light stimuli (arrows) from 3 to 550 photons μm^{-2} (B). Combined fast PIII and PII responses were obtained at the same light intensities in the presence of $BaCl_2$ alone (A) and isolated PII responses to evaluate ON-bipolar cell function were calculated by subtraction of fast PIII from combined fast PIII and PII waveforms (C). Fast PIII (D) and PII (E) amplitude data for db/+ (white squares) and db/db (black squares) mice in normoglycemic conditions (D-E, n=11 for fast PIII and n=4-6 for PII). Averaged fast PIII waveforms normalized to maximal response amplitude at 550 photons μm^{-2} (F), and quantification of the amplification constant (G, n=9, * $p<0.05$, see text for details). Fast PIII (H, n=11, * $p<0.05$) and PII (I, n=4-6) implicit times in db/db (black squares) compared to db/+ (white squares) mice.

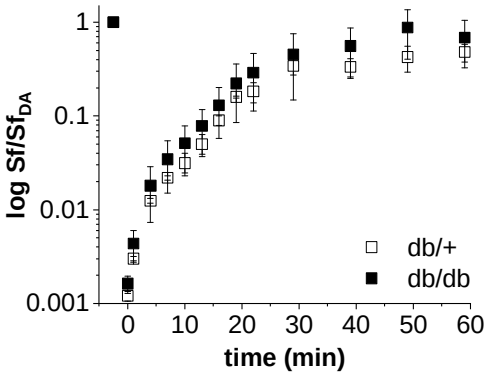


Figure 3: Dark adaptation kinetics of photoreceptors *in vivo* is not altered in diabetes. 90% of the rod photopigments were bleached at 0 min and recovery of b-wave flash sensitivity (normalized to dark adapted rod b-wave sensitivity Sf/Sf_{DA}) was recorded in db/+ (white squares) and db/db (black squares) (n=5 eyes from 3 animals for db/+ and n=2 eyes from 1 animal for db/db).

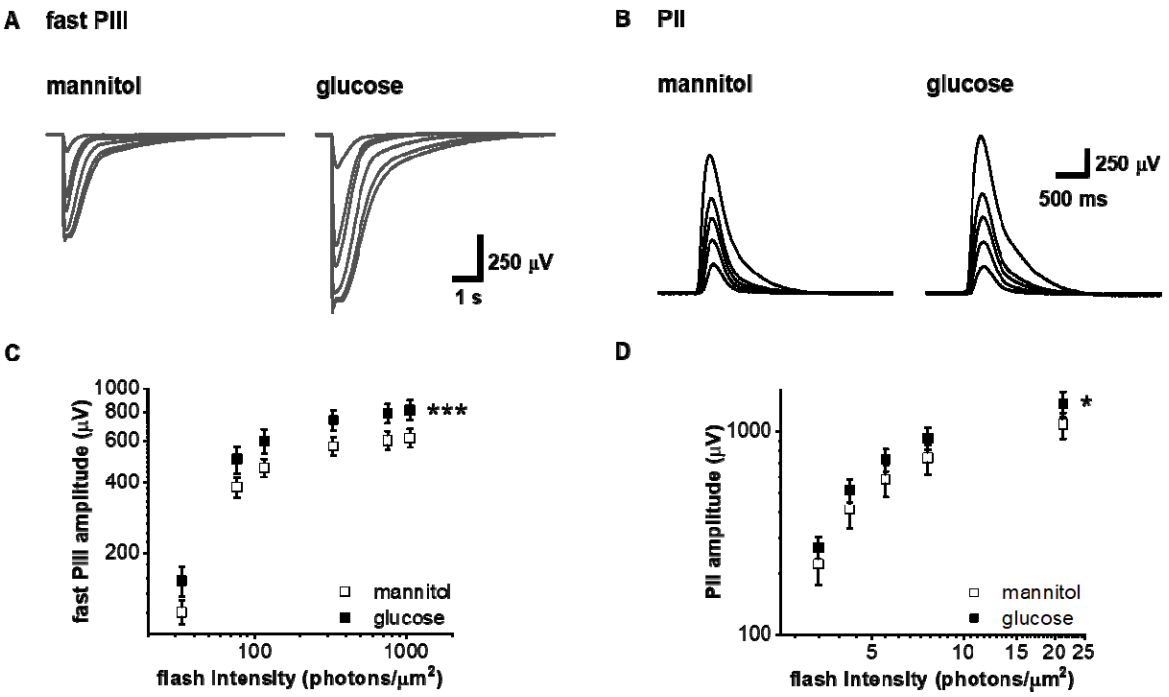


Figure 4: Acute elevation of extracellular glucose increases fast PIII amplitudes of wild-type mice. Representative *ex vivo* ERG fast PIII (A) and PII (B) light response families in normal (iso-osmotic mannitol control, normal glucose + 30 mM mannitol) and after supplementation with 30 mM glucose. Fast PIII (C, *** $p<0.001$, n=7) and PII (D, n=5) amplitude data in normal (mannitol control, white squares) and high glucose (black squares) in C57BL6/J mice.

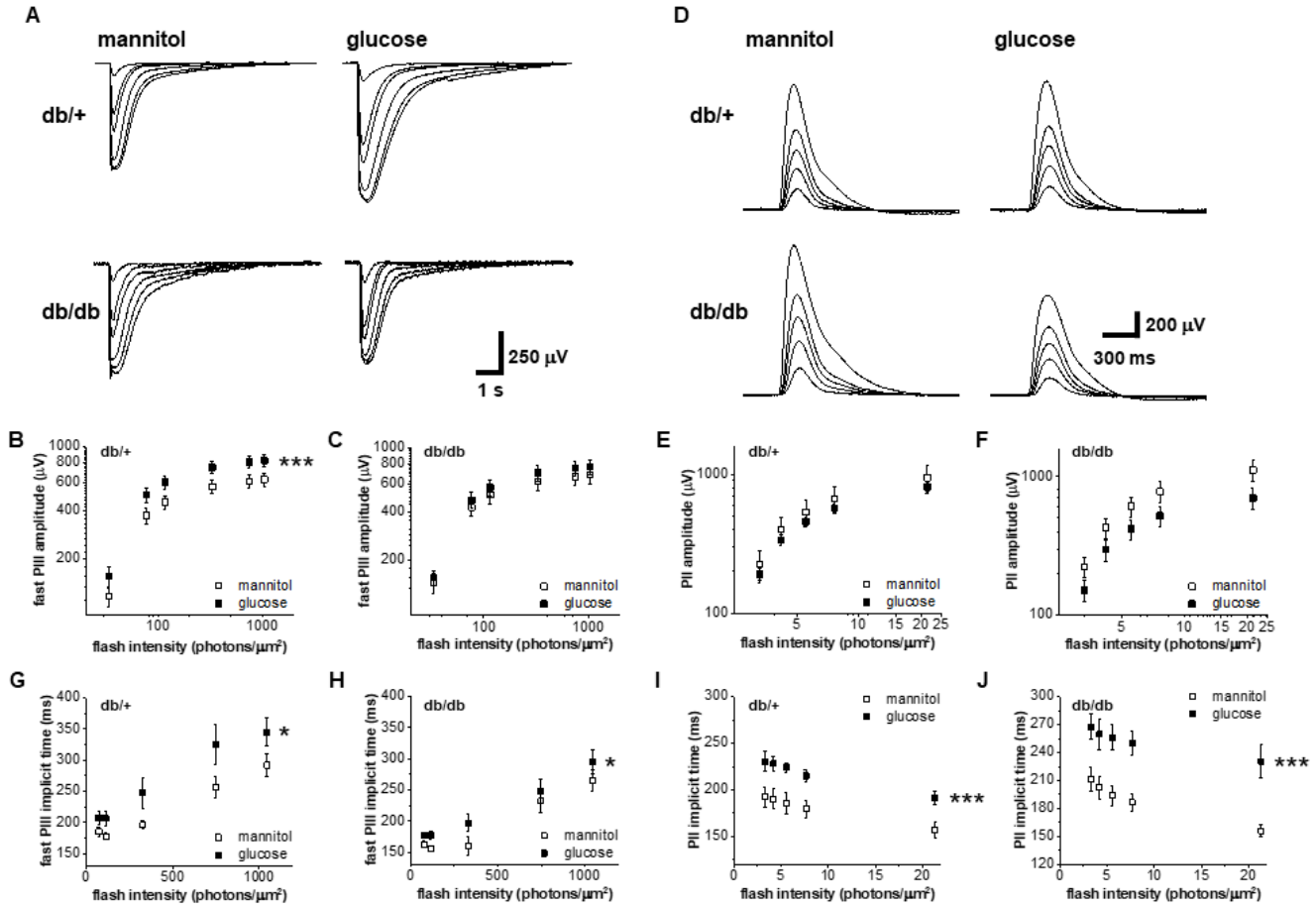
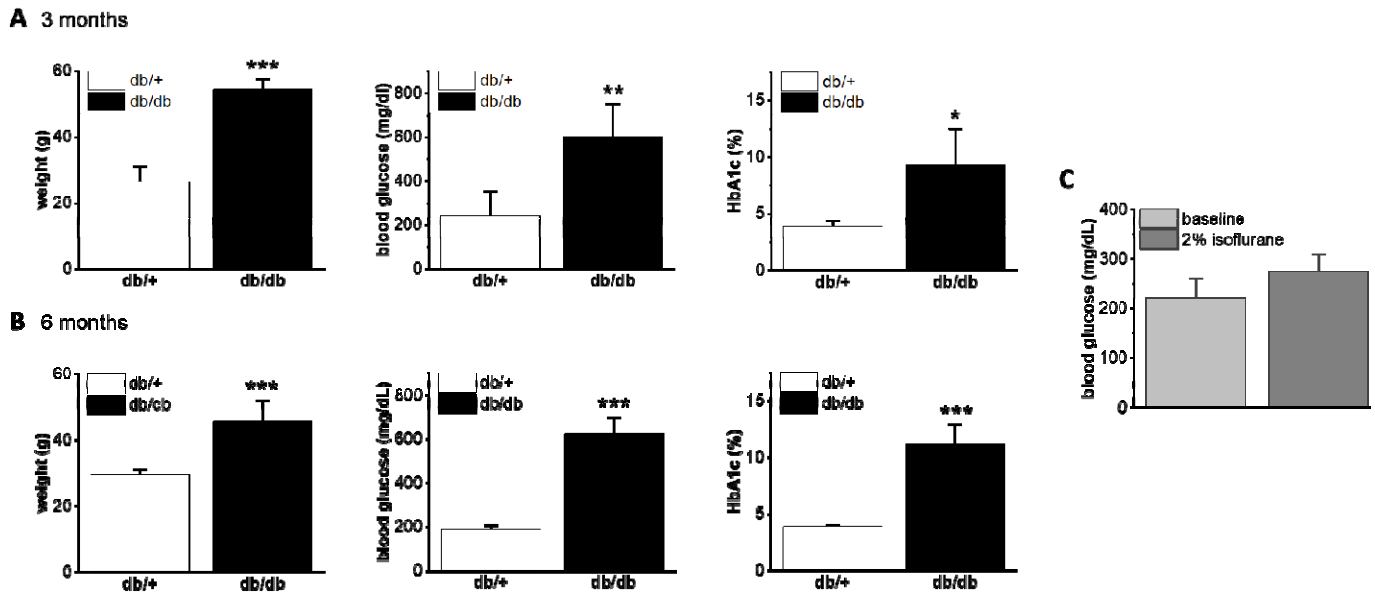


Figure 5: Long-term diabetes modulates the response to acute hyperglycemia in photoreceptors. E*x vivo* ERG fast PIII (A-C, n=11 retinas) and PII (D-F, n=6 db/+ and n=4 db/db retinas) light response families and amplitude data in normal (iso-osmotic mannitol control, normal glucose + 30 mM mannitol, white squares) and after supplementation with 30 mM glucose (black squares) in 6 month old control (db/+, ***p<0.001, B, E) and diabetic (db/db, C, F) mice. Fast PIII (G, H) and PII (I, J) implicit times in normal (white squares) and high (black squares) glucose in db/+ (G, I) and db/db (H, J) mice (G-H, * p<0.05, I-J, *** p<0.001).



Supplemental Figure 1: Weight, blood glucose and HbA1c are increased in diabetic mice. Body weight, blood glucose and HbA1c in db/db mice compared to db/+ mice at 3 months (A, n=3-5, *p<0.05, **p<0.01, ***p<0.001) and 6 months of age (B, n=16-21, ***p<0.001). Blood glucose in C57BL/6J mice before and during 2% isoflurane inhalation (C, n=5)