Loss of Synaptic Connectivity, Particularly in Second Order Neurons Is a Key Feature of Diabetic Retinal Neuropathy in the Ins2Akita Mouse


Published in:
PloS one

Document Version:
Publisher's PDF, also known as Version of record

Queen's University Belfast - Research Portal:
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Download date:19. Jun. 2017
Introduction

Diabetic retinopathy (DR) is a sight-threatening complication of diabetes and the leading cause of blindness in working populations [1]. Neural retinal degeneration together with microvascular dysfunction, constitute the key pathologies of DR [2,3]. Vascular complications (i.e. diabetic retinal vasculopathy) comprise a well-defined set of changes, which progress from capillary degeneration and vascular leakage in non-proliferative DR to retinal neovascularization in proliferative DR [4,5]. Features of diabetic retinal vasculopathy including microaneurysms, intraretinal hemorrhages and neovascularization are readily detectable in fundus examination. Visual impairment in DR is often associated with vascular dysfunction (macular oedema, vitreous hemorrhage or tractional retinal detachment). As a result, DR-related vasculopathy has been researched extensively. Although diabetes related retinal neural degeneration (diabetic retinal neuropathy) has been recognized for over five decades [6,7], the detailed neural pathology remains ill-defined. With advancements in the management of diabetic macular oedema and proliferative DR, further vision improvement will rely upon the development of neuroprotective strategies. From this perspective, a clearer understanding of how the neurosensory retina is affected under diabetic conditions will be crucial.

Diabetic retinal neuropathy has been investigated in humans and various animal models. The majority of studies have reported a progressive loss of inner retinal neurons, including amacrine and retinal ganglion cells (RGCs) [8]. This degeneration is likely to be responsible for the early electrophysiological deficits in DR [9,10]. A growing body of evidence suggests that neuropathy in DR may also involve other neural elements. For example, impairment in colour vision (in particular blue-sensitive defects) and decreased contrast sensitivity constitute an early feature characterizing visual dysfunction in DR [11,12]. Studies in animal models have revealed some cellular components of diabetic retinal neuropathy. In diabetic rodents for example, photoreceptor death [13,14], reduced expression of presynaptic photoreceptor proteins [15], abnormalities in horizontal and bipolar cell synaptic terminals [13,16] and dendritic remodeling of RGCs [17] were observed.

In the present study, we characterized retinal neuropathy at different stages of disease in the Ins2Akita mouse (model of type-1 diabetes). Previous studies have shown that Ins2Akita mice develop various early DR-related retinal abnormalities, including increased vascular permeability, RGCs apoptosis, reduced retinal thickness [18,19] and abnormal electroretinographic responses [20]. We found that the Ins2Akita mouse presents a variety of retinal neuropathic lesions including the degeneration of cone photoreceptors after a relatively short disease duration (3-month old), and loss of synaptic structures in second order neurons and reduced number of amacrine cells and RGCs at the later stages of the disease (9-month old).
Materials and Methods

Animals
Male heterozygous Ins2Akita mice (Originally purchased from Jackson Laboratory, Bar Harbor, USA, stock number 003548) of C57BL/6j background and age-matched non-diabetic siblings were used in the study. The Ins2Akita mouse develops robust hyperglycemia by 4.5 weeks of age [21]. Only males were used in this study. Experimental mice were generated by mating Ins2Akita heterozygous males with C57BL/6j females, and diabetes confirmed in 6-week old littermates by blood glucose measurements (>250 mg/dL; the majority of Ins2Akita mice had ≥540 mg/dL – above the maximal detectable level of the glucometer; non diabetic siblings had 151±19.8 mg/dL). All mice were housed in standard pathogen-free animal housing rooms with 12/12-hour light/dark cycle and free access to food and water. All procedures were conducted under the regulation of the UK Home Office Animals (Scientific Procedures) Act 1986 and approved by the Ethical Review Committee of Queen’s University Belfast (Project Licence Number: PPL 2664). The study was conducted in compliance with the Association for Research in Vision & Ophthalmology Statement for the Use of Animals in Ophthalmology and Vision Research.

Electroretinography
Scotopic electroretinogram (ERG) responses were evaluated in Ins2Akita and control mice at 3, 6 and 9 months of age (n = 6 mice per strain/age). Mice were dark-adapted overnight and all procedures were conducted under dim-red light (<1 Lux). Briefly, mice were anesthetized with an intraperitoneal injection of ketamine hydrochloride (60 mg/kg; Fort George Animal Centre, Southampton, UK) and xylazine (5 mg/kg; Pharmacia & Veterinary Products, Kiel, Germany) and pupils dilated using 1% tropicamide and 2.5% phenylephrine (Chauvin, Essex, UK). The head was secured with a stereotaxic frame and the body temperature maintained with a homeothermic pad at 38°C (Kobayashi Healthcare, London, UK). ERG was recorded using mouse corneal ERG electrodes in response to single white light stimuli of 15 seconds (0.08, 0.25, 0.8 cd/m²), 30 seconds (0.08, 0.25, 8 cd/m²) or 60 seconds (0.8 cd/m²). Oscillatory potentials (OPs) were obtained by high band filtering at 75 Hz and recorded using 25 cd/m² flash intensity. The following ERG parameters were measured: a-wave amplitude (baseline to negative a-wave peak), b-wave amplitude (a-wave peak to positive b-wave peak), a-wave implicit time (from stimulus onset to negative a-wave peak), b-wave implicit time (from stimulus onset to positive b-wave peak), OPs amplitude (the summed amplitude of wavelets 2–5) and OPs implicit time (the summed implicit times of wavelets 2–5). The b/a-wave amplitude ratio was calculated at the highest flash intensities (0.8, 2.5, 8, 25 cd/m²).

Photopic ERG responses were also evaluated in Ins2Akita and control mice at 3 and 9 months of age (n = 5 mice per strain/age). Three light intensities (2.5, 8 and 25 cd/m²) were used to stimulate the light-adapted mice (on a 30 cd/m² background light). The amplitude and implicit time of the photopic b-wave were averaged from 5 responses at each light intensity level.

Clinical investigations
Animals were anesthetized by isoflurane inhalation (Merial; Animal Health Ltd; Essex, UK) and pupils dilated as described above. A topic endoscopic fundus imaging system was used to obtain fundus images as described previously [22,23]. Spectral Domain Optical Coherence Tomography (SD-OCT) was conducted using the Spectralis Heidelberg OCT system (Heidelberg Engineering, Heidelberg, Germany).

Retinal thickness analysis
Retinal thickness was measured in Ins2Akita and control mice at 3, 6 and 9 months of age (n = 6 animals per strain/age) from the SD-OCT images. Three types of measures were conducted: 1) total retinal thickness: from the nerve fibre layer (NFL) to the outer margin of photoreceptor outer segments (OS); 2) inner retinal thickness: from the NFL to the margin between the inner nuclear layer (INL) and outer plexiform layer (OPL); 3) outer retinal thickness: from the INL/OPL margin to the outer margin of OS. The SD-OCT layers were defined according to previous investigations in the mouse retina [24,25]. Retinal thickness was measured at 300, 600 and 900 μm eccentricities from the optic disc (OD) in dorsal-ventral and nasal-temporal sectors. Isodensity maps of average retinal thickness were constructed using Sigma Plot v12.0 (Systat Software, London, UK).

Immunohistochemistry
Mice were sacrificed by CO2 inhalation and eyes dissected and fixed in 2% paraformaldehyde for 2 h. Eyes were then processed for immunohistochemistry as previously described [26,27]. Briefly, 14 μm-thick retinal cryosections were incubated overnight (4°C) with primary antibodies (Table 1) diluted in 0.5% TritonX-100 with 10% normal donkey serum in PBS. Sections were then incubated for 2 h at room temperature with appropriate fluorophore-conjugated secondary antibodies. All sections were cover-slipped with Vectashield-DAPI (Vector Labs, Burlingame, CA) and examined by confocal microscopy (C1 Nikon Eclipse TE200-U, Nikon UK Ltd, Surrey, UK).

Retinal morphometry
Confocal images were used to quantify retinal neurons and synaptic structures in Ins2Akita and control mice at 3, 6 and 9 months of age (n = 3 animals per strain/age). Data was acquired from 3 sections per eye at the same retinal eccentricities (0.6, 1.2 and 1.8 mm from the OD). Confocal settings remained unchanged in all studies during image acquisition. Images were analysed using ImageJ software (National Institutes of Health, Bethesda, MD).

Using the listed panel of antibodies we carried out the following analyses: 1) rod and cone photoreceptor density (rods estimated as the difference between DAPI+ nuclei at the outer nuclear layer and the number of cone somata), 2) photoreceptor synaptic ribbon density, 3) horizontal, rod-bipolar and cone-bipolar cell density, 4) horizontal and rod-bipolar cell dendritic spines density, 5) rod- and cone-bipolar dendritic length (measured from the apical border of the cell soma), 6) rod- and cone-bipolar cell axon bouton density, 7) GABAergic and glycineergic amacrine cell density, and 8) RGCs density. At least 20 retinal images per strain/age were analyzed and values averaged and normalized to 100 μm retinal length. The density of dendritic spines in horizontal cells was measured in 4 cells per image. The density of dendritic spines and axon boutons in rod-bipolar cells and the dendritic length of rod- or cone-bipolar cells were measured in 20 cells per image.
Data analysis

ERG responses (scotopic a-wave, b-wave and photopic b-wave), and morphometry data were analysed using 2-way ANOVA (two strains of mice and different ages), followed by post-hoc Bonferroni’s pairwise comparisons. OPs responses and retinal thickness were analysed using unpaired Student’s t-test. Data were expressed as mean ± SEM (ERG) or ± SD (retinal thickness, retinal morphometry). P<0.05 was considered statistically significant.

Table 1. Primary antibodies used in the study.

<table>
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<th>Antigen</th>
<th>Antiserum</th>
<th>Dilution</th>
<th>Source</th>
<th>Retinal localization</th>
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<td>Chemicon</td>
<td>Rod photoreceptors</td>
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<td>Cone arrestin</td>
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<td>Enzo</td>
<td>Synaptic ribbons</td>
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<td>Glycine Transporter-1</td>
<td>goat anti-glyT1</td>
<td>1:3000</td>
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<td>Glycinergic amacrine cells</td>
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<td>goat anti-Bm3a</td>
<td>1:500</td>
<td>Santa Cruz</td>
<td>Retinal ganglion cells</td>
</tr>
</tbody>
</table>

doi:10.1371/journal.pone.0097970.t001

Figure 1. Abnormal scotopic electroretinogram (ERG) responses in the Ins2Akita mouse. (A) Representative scotopic ERG responses obtained from control (black) and Ins2Akita mice (orange) of different ages. (B) The amplitude (μV) and implicit time (milliseconds, ms) of the dark adapted a- and b-waves in control and Ins2Akita mice. (C) The b/a-wave ratio in different ages of control and Ins2Akita mice. (D) The amplitude and implicit time of oscillatory potentials in control and Ins2Akita mice. (B-D) n = 6 mice per strain/age, *P<0.05, **P<0.01, ***P<0.001. 2-way ANOVA (a- and b-wave responses), Student’s t-test (OPs responses).

doi:10.1371/journal.pone.0097970.g001
Results

Abnormal ERG responses in the Ins2Akita mouse

Scotopic ERG analysis in 3-month old Ins2Akita mice showed a slight but statistically significant reduction in the b-wave amplitude in the higher flash intensity (25 cd s/m²) compared to age-matched controls (Figure 1A-B). Interestingly, 6-month old Ins2Akita mice had significantly faster b-wave responses (0.08 and 0.25 cd s/m²) b-wave implicit time, Figure 1B) compared to control mice. By 9-month old, the Ins2Akita mouse displayed smaller amplitude and increased implicit times in both a-wave (reduced amplitude between 0.8–25 cd s/m² flash light; increased implicit time at 0.025 cd s/m² flash light) and b-wave (reduced amplitude between 0.025–25 cd s/m² flash light; increased implicit time between 0.025–25 cd s/m²) responses. Further analysis using b/a-wave ratio revealed a strikingly disproportionate reduction in the b- and a-wave amplitudes (p<0.001; Figure 1C), suggesting that the abnormal drop in b-wave amplitude may arise from disorders at the distal retina rather than from photoreceptors.

OPs responses were evaluated to assess the functional activity of amacrine cells (Figure 1D). No changes were observed in the amplitude or implicit times of OPs by 3 months of age. An apparent increase in the OPs amplitude was evident in 6-month old Ins2Akita mice, but failed to reach statistical significance. Closer inspection of these traces, however, revealed an increase in the fourth OP wavelet (p<0.05; figure S1A). OPs implicit times appeared normal at this stage (Figure 1D). By 9-month old, OPs responses were significantly reduced and delayed in the Ins2Akita mouse (p<0.001; Figure 1D and figure S1B).

No differences were observed in the photopic b-wave amplitude between Ins2Akita mice and sibling controls of 3 months old (figure S1C). However, by 9 months of age, the amplitude of the photopic b-wave was significantly reduced in 25 cd s/m² flash light (p<0.01; figure S1D). The implicit time of the photopic b-wave remained similar to age-matched controls in both age groups (data not shown).

Retinal degeneration in the Ins2Akita mouse

SD-OCT was carried out in 3-, 6-, and 9-month old control and Ins2Akita mice (Figure 2). As shown by isodensity maps, there was an age-dependent reduction in the overall retinal thickness (OS-NFL) of Ins2Akita mice but not age-matched controls (Figure 2B). This reduction occurred in all retinal sectors for all the eccentricities analysed (Figure 2B-C). Further analysis of outer (OS-OPL) and inner (INL-NFL) retinal thickness revealed that at 3 months of age, only the outer retina was affected in the Ins2Akita mouse (p<0.001, Figure 2D). From 3–6 months, no further reduction was observed in the outer retina, but a significant reduction was detected in the inner retina (p<0.001). From 6–9 months, the thickness of both outer and inner retina was further reduced (p<0.001) compared to non-diabetic controls.

Despite a significant reduction in retinal thickness, other human DR related lesions (e.g. microaneurysm, exudates) were not detected in fundus imaging and SD-OCT investigation up to 9 months of age in Ins2Akita mice (Figure 2E-J).

Characterization of diabetic retinal neuropathy in the Ins2Akita mouse

Photoreceptor cells. Rod and cone photoreceptors were evaluated by using antisera against rhodopsin and cone-arrestin respectively (Figure 3). Control and Ins2Akita mice showed normal expression of rhodopsin in rod outer segments up to 9 months of age (Figure 3A-F). No significant changes were observed in the
total number of photoreceptors and rods from 3-9 months between control and Ins2Akita mice (Figure 3G). Interestingly, the number of cone photoreceptors was 10% less in 3-month old Ins2Akita compared to age-matched controls (p < 0.05) and further decreased by 9 months of age (p < 0.01; Figure 3H). Thus, cone but not rod photoreceptors selectively degenerate from an early stage in Ins2Akita mice.

**Outer plexiform layer connectivity**

We next investigated synaptic connectivity at the outer retina by studying pre- and post-synaptic processes at the OPL (Figure 4, 5).
Immunostaining of bassoon revealed synaptic ribbons in rod and cone cell terminals (Figure 4A). Many of the presynaptic ribbons displayed a typical horseshoe-like morphology in Ins2Akita mice (Figure 4B-C). However, despite the absence of morphological anomalies, a 25% reduction of photoreceptor synaptic ribbons was detected in 9-month old Ins2Akita (p<0.01, Figure 4M). This reduction did not correlate with a proportional loss of total photoreceptors at this stage (Figure 3G), suggesting additional damages to the photoreceptor synaptic complex.

Photoreceptor postsynaptic elements were identified by immunostaining of secretagogin (cone-bipolar cells) (Figure 4D-F), PKCα (rod-bipolar cells) (Figure 4G-I) and calbindin (horizontal cells) (Figure 4J-L). An age-dependent increase in the length of cone- and rod-bipolar dendritic projections (Figure 4N, 4O and figure S2) was observed in non-diabetic controls. By contrast, there was a significant reduction of in the length of both cone-bipolar (15%, p<0.05) and rod-bipolar dendritic projections (20%, p<0.001) in 9-month old Ins2Akita mice (Figure 4N, 4O and Figure S2). The number of horizontal cell dendritic tips (postsynaptic boutons) was also reduced in this stage (Figure 4J-L).

Double immunostaining against bassoon and PKCα or calbindin showed the close association between photoreceptor presynaptic terminals and postsynaptic boutons of rod-bipolar or horizontal cells in control and 6-month old Ins2Akita mice (Figure 5A-B, 5E-F). However, the absence of paired connectivity, predominately due to a lack of dendritic boutons in second-order neurons, was observed in 9-month old Ins2Akita mice (Figure 5C, G). Quantitative analysis revealed a 70% reduction (p<0.001) of both rod-bipolar (Figure 5D) and horizontal cell dendritic tips (Figure 5H) at this stage compared to age-matched controls.

Changes in bipolar and horizontal cells

The fact that postsynaptic dendrite reduction outweighs presynaptic ribbon loss in the Ins2Akita mouse suggests that the impairment in OPL connectivity may result from the loss of second-order neurons. Thus, we evaluated cell densities of horizontal, cone-bipolar and rod-bipolar cells (Figure 6). Surprisingly, no differences were observed in the density of horizontal, cone-bipolar or rod-bipolar cells between Ins2Akita mice and controls in all age groups (Figure 6E, J). Moreover, quantitative analysis of the total number of cone-bipolar cell axon terminals (Fig. 6K-L) revealed no difference between control and Ins2Akita mice (data not shown). However, a progressive reduction of rod-bipolar cell axonal boutons was observed in the Ins2Akita mouse from 6 months onwards (Figure 6M-O; 6 months, p<0.05; 9 months, p<0.01).

Changes in amacrine and retinal ganglion cells

To further understand retinal neuropathy in Ins2Akita mice, we examined amacrine cells and RGCs (Fig. 7). Amacrine cells of the INL were assessed by using antibody against GABA or GlyT1 (GABAergic or glycinergic subtypes respectively), since most amacrine cells express one of these neurotransmitters [28,29]. No differences were observed in either GABAergic or glycinergic

Figure 4. Synaptic elements in the OPL of control and Ins2Akita mice. (A-C) Photoreceptor synaptic ribbons immunostained with bassoon in 9-month old control and 6- and 9-month old Ins2Akita mice. Immunostaining of cone-bipolar (secretagogin) (D-F) and rod-bipolar (PKCα) (G-I) and horizontal cells (calbindin) (J-L) in 9-month old control and 6- and 9-month old Ins2Akita mice. Normal dendritic arbors of cone-bipolar, rod-bipolar and horizontal cells at the OPL (arrowheads). Loss of dendritic complexity in cone-bipolar, rod-bipolar and horizontal cells in 9-month old Ins2Akita mice (Figure 5C, predominately due to a lack of dendritic boutons in second-order (Figure 5A-B, 5E-F). However, despite the absence of morphological anomalies, a 25% reduction of photoreceptor synaptic ribbons was detected in 9-month old Ins2Akita (p<0.01, Figure 4M). This reduction did not correlate with a proportional loss of total photoreceptors at this stage (Figure 3G), suggesting additional damages to the photoreceptor synaptic complex.

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Figure 5. Synaptic structure in the OPL of control and Ins2Akita mice. Photomicrographs of retinal sections double-labelled for bassoon (red) with PKCα (A-C) or calbindin (E-G) in 9-month control and 6- and 9-month old Ins2Akita mice. Absence of paired connectivity between photoreceptor terminals and second-order neurons in 9-month old Ins2Akita mice (Figure 6). Moreover, quantitative analysis of the total number of cone-bipolar cell axon terminals (Fig. 6K-L) revealed no difference between control and Ins2Akita mice (data not shown). However, a progressive reduction of rod-bipolar cell axonal boutons was observed in the Ins2Akita mouse from 6 months onwards (Figure 6M-O; 6 months, p<0.05; 9 months, p<0.01).

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amine cells between 3-month old controls and Ins2Akita mice (Figure 7I-J). Although the number of GABAergic amacrine cells remained similar to controls by 6 months of age, GABA immunoreactivity was increased at the IPL (Figure 7C). By 9 months, both the density of GABAergic amacrine cells and GABA immunoreactivity were reduced in Ins2Akita mice compared to controls (Figure 7D, 7I). The morphology of glycinergic amacrine cells remained unchanged in control and Ins2Akita mice of all ages (Figure 7E-F). However, there was an age-dependent reduction in the cell density in both strains of mice, although the reduction was greater in the Ins2Akita mouse (Figure 7J). In addition, we observed a 20% reduction in the number of Brn3a+ RGCs in 9-month old Ins2Akita mice compared to age-matched controls (p<0.05, Figure 7G-H, K).

Discussion

This study provides a detailed time-course analysis of the retinal neurodegenerative changes occurring in Ins2Akita mice. Mild scotopic ERG abnormalities and cone photoreceptor degeneration were observed at the early stages of diabetes, followed by significant ERG defects, severe synaptic structure disruption (particularly at the OPL) and loss of amacrine cells and RGCs after long-term diabetes.

Early thinning of the neurosensory retina occurs predominately at the outer retinal layers in Ins2Akita mice, whilst the inner retina progressively degenerates from 3 months onwards. Recent studies reported no changes in retinal thickness in 14–25 weeks old Ins2Akita mice by OCT investigation [30,31]. What causes the discrepancy is not clear, but it may be related to different approaches in image analysis. The total retinal thickness in those studies was automatically measured between the ILM and Bruch’s membrane (BM) using the Heidelberg Eye Explore system. The BM in mouse eyes is not always as clear as other retinal layers. Therefore, we used the OS as a landmark to measure the thickness manually. Our results are supported by previous studies using post-mortem histological investigations [19,32], which demonstrated significant retinal thinning by 6 months of age. The results are also supported by other data from our study, including neuronal degeneration and abnormal ERG responses.

Diabetes can promote photoreceptor degeneration. Unlike the STZ-induced diabetic rat, in which both cones and rods undergo degeneration [13,14], the Ins2Akita mouse only develops cone degeneration starting from 3 months and progresses further by 9 months old (which was supported by the abnormal photopic ERG responses observed in this study (Figure S1D) as well as in previous studies [20,33]). The lack of significant changes in photopic ERG in 3 months old Ins2Akita mice (compared to a significant loss of cone photoreceptors at this stage) may suggest that the traditional white-flash ERG is not sensitive enough to detect this level of neurodegeneration. The absence of significant anomalies in rod photoreceptors together with the large rod/cone ratio (~30:1) in the mouse retina [34,35] may explain the relatively minor changes in scotopic a-wave amplitudes occurring in Ins2Akita mice. Cone dysfunctions, including abnormal photopic ERG [36] and impaired color vision sensitivities [12,37] have been observed in early stages of human diabetes (before the development of clinically detectable DR). In this regard, the Ins2Akita mouse is
an excellent model that recapitulates this early neuropathological feature of human DR. Why cone photoreceptors selectively degenerate in the diabetic retina remains elusive. A previous study has shown that cone but not rod metabolism is enhanced with increasing glucose levels in diabetes [38], which may result in excessive oxidative stress to the cells.

Another important observation of this study is the disruption of synaptic structures at the OPL after long-term diabetes, in particular those related to postsynaptic second-order neurons. This is supported by functional ERG changes (significant reduction of the b/a wave ratio) and greater depletion of postsynaptic dendrites compared to presynaptic ribbons. It should be pointed out that the reduced immunoreactivity of calbindin and PKCa at the OPL is likely due to the loss of dendritic and synaptic structures rather than the lack of protein expression, as calbindin and PKCa proteins were detected at high levels in other compartments of horizontal and rod-bipolar cells. Further study using immuno-electron microscopy may help to confirm this. Our results suggest that long-term diabetes can damage synaptic connectivity in the OPL, by preferentially affecting synaptic structures in second-order neurons. Previous studies have observed diverse OPL anomalies in the diabetic eye, although the majority of these are related to photoreceptor presynaptic structures. For example, down-regulation of photoreceptor presynaptic proteins has been observed in rodent models of diabetes [13,39], whilst mitochondria degeneration was reported in OPL-terminals of photoreceptors and second-order neurons of STZ-diabetic rats [13]. Gao et al. [40] reported a reduction in OPL thickness and OCT-reflectance in patients with mild DR. It has been proposed that the collapse of synaptic structures may be related to the generation of reactive oxygen species [41,15], a condition that is particularly aggravated in 9-month old Ins2Akita mice [33]. Furthermore, previous studies have shown that hyperglycemic stress can also impair synaptic function in the nervous system [42,43]. The disruption of retinal synaptic structure in the OPL may be related to the toxic effect of hyperglycemia and oxidative stress to second-order neurons.

DR related retinal neuropathy has previously been linked to the degeneration and dysfunction of inner retinal neurons, including amacrine cells and RGCs [17,19,44]. We detected a significant reduction of amacrine cells and RGCs in 9 months old Ins2Akita mice. Previous studies by Gastinger et al. [17,18] reported a loss of cholinergic and dopaminergic amacrine cells in 6 months old Ins2Akita and a significant degeneration of RGCs in the peripheral retina after 3 months of diabetes. The discrepancy may be related to different immunohistochemical approaches (retinal flatmount versus retinal section) and different cell markers used for morphometry. In the current study, the changes in GABA+ amacrine cells in Ins2Akita mice correlate to OPs electrophysiological responses. OPs are known to be a useful tool for evaluating

Figure 7. Amacrine and retinal ganglion cell loss in the Ins2Akita mouse. Inner retina photomicrographs of GABA (A-D), GlyT1 (E, F) or Brn3a immunoreactivities (G, H) in 9-month old control and 3-, 6- and 9-month old Ins2Akita mice. Quantitative analysis of INL-GABAergic amacrine cells (box1) (I), INL-glycinergic amacrine cells (box2) (J) and retinal ganglion cells (box3) (K) in control and Ins2Akita mice at different ages. (I-K) n=20 retinal images per strain/age, *P<0.05, **P<0.01. 2-way ANOVA. INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bar: 20 μm. doi:10.1371/journal.pone.0097970.g007

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inner retinal activity, particularly amacrine cells [45,46], and has been used as a reliable parameter to predict the progression of DR [47]. We reported increased OPs responses in Ins2^Akita^ mice by 6 months of age. The enhanced OPs may be related to abnormal expression of GABA in the IPL. A similar change was reported in STZ-diabetic female rats [10]. The turnover of GABA and GABA<sub>e</sub> receptors is impaired in the diabetic retina [10,48], and therefore, GABA<sub>e</sub>ergic signalling may be compromised. By 9 months of age, amacrine cells were significantly depleted and OPs substantially attenuated.

In summary, we show for the first time that in addition to inner retinal neuronal degeneration, the Ins2^Akita^ mouse presents a variety of retinal neuropathies that are relevant to visual dysfunction in human diabetic patients, including the early reduction of cone photoreceptors, and the disruption of synaptic structures in photoreceptors and second-order neurons. Our findings strongly suggest that the Ins2^Akita^ mouse is a good model for studying diabetic retinal neuropathy.

**Supporting Information**

**Figure S1** Scotopic Oscillatory potential (OPs) and photopic b-wave responses in the Ins2^Akita^ mouse. (A, B) Representative OPs responses obtained from 6-month (A) and 9-month old (B), control (black) and Ins2^Akita^ (orange) mice. The 6 individual OPs wavelets are enumerated. (C, D) The amplitude (mV) of the photopic b-wave in control and Ins2^Akita^ mice at 3 (C) and 9 months (D) of age. (C-D) n = 5 mice per strain/age, **P< 0.01. 2-way ANOVA.**

**Figure S2** Dendritic projections of rod-bipolar and cone-bipolar cells in control and Ins2^Akita^ mice. Confocal photomicrographs of vertical retinal sections processed for PKC<sub>α</sub> (A-F) or secretagogin immunoreactivity (G-L) in control and Ins2^Akita^ retinas at 3, 6 and 9 months of age. Control retinas present an age-dependent increment in the length of cone- and rod-bipolar dendritic projections. OPL, outer plexiform layer; INL, inner nuclear layer. Scale bar: 10 μm.**

**Acknowledgments**

The authors are grateful to Dr Timothy Curtis for critically reading the manuscript.

**Author Contributions**

Conceived and designed the experiments: JRH MC HX. Performed the experiments: JRH RGP. Analyzed the data: JRH RGP. Contributed reagents/materials/analysis tools: JRH MC HX. Wrote the paper: JRH HX.

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