Neural Apoptosis in the Retina during Experimental and Human Diabetes
Early Onset and Effect of Insulin
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Introduction
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1. Abbreviations used in this paper: STZ, streptozotocin; TUNEL-FITC, terminal dUTP nick end labeling-fluorescein-isothiocyanate; TUNEL-HRP, terminal dUTP nick end labeling-horseradish peroxidase.

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Introduction
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The pathologic vascular lesions associated with diabetic retinopathy have been described using the trypsin digest prepara-
onset of diabetes; and (2) which cells, other than vascular cells, become apoptotic? The following study quantifies apoptosis in the retinas of streptozotocin diabetic rats and in postmortem retinas of humans with diabetes. We report that neural cell apoptosis is increased after 1 mo of diabetes and remains elevated over a 12-mo period. Furthermore, retinal apoptosis is reduced by insulin. There are overall reductions in the thickness of the inner layers of the retina, accompanied by a reduction in the number of ganglion cells in rat retinas after long-term experimental diabetes. These findings reveal that retinal neuron and glial cell degeneration occurs early on in the course of diabetes and should be considered an important component of the pathology of diabetic retinopathy.

Methods

Animals. Male Sprague-Dawley rats weighing 150–175 g were purchased from Charles River Laboratories (Wilmington, MA) and housed in the Penn State University College of Medicine animal facility in accordance with the Institutional Animal Care and Use Committee guidelines, and the Association of Research in Vision and Ophthalmology Resolution on the Care and Use of Laboratory Animals. All rats were group housed in suspended wire bottom cages with ad libitum food and water and under a normal 12-h light/dark schedule. Diabetes was induced by a single tail vein injection of streptozotocin (STZ; Sigma, St. Louis, MO) (65 mg/kg freshly dissolved in citrate buffer, pH 4.5) and confirmed three days later by urine glucose assay. To control for non-diabetic mortality, half the rats were given an equal volume of citrate buffer only. Rats that died within 48 h of STZ injection were excluded from the study. Diabetes was monitored by tail vein capillary blood glucose measurements, using a Lifescan meter method (Glycotest II kit; Pierce Chemical Co., Rockford, IL) at sacrifice. All rats were weighed weekly. Long-term diabetic rats were injected with 2 U of insulin once a week after the second month of diabetes (subcutaneous Humulin Ultralente; Eli Lilly, Indianapolis, IN) to stabilize weight loss. Insulin-controlled diabetic rats were given small, solid phase subcutaneous implants (Linplant; Linshin, Canada) to deliver 2–4 U of bovine insulin/24 h. The implants were delivered below the skin through an 18G needle, under light anesthesia. No sutures were required and no bleeding or infection occurred. Rats were sacrificed under deep ether anesthesia followed by decapitation, and both eyes were removed and aged-matched control rats were analyzed. 16 sections (6 µm thick) of each eye were collected from the posterior portion of each eye. Sections were processed for paraffin embedding.

Histology and image analysis. Eyes from 7.5 mo diabetic (n = 6) and aged-matched control rats were analyzed. 16 sections (6 µm) were collected at the midportion of each eye at approximately 40 µm intervals were stained with thionin, a Nissl stain, to identify neuronal cell bodies. Images were captured using Optimas software on a PC computer linked to a high resolution video camera (DXC-960MD; Sony) mounted on a microscope (BH-2; Olympus, Melville, NY). Five visual fields were sampled from the posterior portion of each retina using a 40X objective (each visual field representing a 340 µm length of retina). Within each visual field, the thickness of the inner plexiform layer, inner nuclear layer, and entire retina (excluding photoreceptors) was measured by the method of Hughes (25). To compensate for any bias due to skewed sectioning angle (i.e., deviation from 90°), all measurements were expressed relative to the thickness of the choroid in the respective section. The number of ganglion cells was also quantified in each visual field and the total count for the five sampled fields was expressed per 100 µm. Cells were categorized as ganglion cells only if they appeared in the ganglion cell layer and had large, round cell bodies containing positively-stained Nissl substance.

Terminal transferase dUTP nick end labeling (TUNEL). TUNEL was performed with horseradish peroxidase detection (TUNEL-HRP) in whole-mount retinas (26, 27), or with fluorescein detection (TUNEL-FITC) in 10 µm cryostat sections. Isolated retinas were fixed in normal buffered formalin for 10 min at room temperature, and flattened mounted on microscope slides coated with 2% 3-aminopropyltriethoxysilane in humidified boxes, at 4°C overnight, before storing at −20°C. Before TUNEL-HRP staining, the retinas were rehydrated in PBS and fixed in 4% paraformaldehyde for 10 min. The tissue was then dehydrated through graded alcohols, and defatted in xylene overnight to allow better penetration across the inner limiting membrane of the retina (27). The following day, the sections were rehydrated and permeabilized with 0.3% Triton for 15 min, and digested with proteinase K (20 µg/ml, Sigma) for 30 min. Endogenous peroxidase was quenched with 3% hydrogen peroxide for 10 min. TUNEL-HRP staining was performed with the horseradish peroxidase Apoptag kit (Oncor Inc., Gaithersburg, MD), following the manufacturer’s instructions. This kit uses terminal transferase to tail single or double strand 3’-OH ends of DNA with digoxigenin-11-dUTP and dATP, which is then recognized with an HRP-bound antidigoxigenin antibody. The HRP was reacted with 0.05% 3,3’-diaminobenzidine tetrahydrochloride (Gibco BRL, Gaithersburg, MD) and 0.03% hydrogen peroxide (Sigma, St. Louis, MO) to develop a brown precipitate that was easily observed in the whole-mount retinas. Each staining included a number of controls treated identical to the experimental tissue. The first positive control for TUNEL-HRP staining was a section of paraffin-embedded ventral prostate from a castrated rat (large-scale apoptosis occurs in the ventral prostate two days after castration) (28). This was compared to a similar section from an uncastrated adult rat. To provide a negative control, we also evaluated prostate sections from castrated rats by omitting terminal transferase. The second positive control, a whole-mounted ischemic retina, served as a comparison for neuronal apoptosis in whole-mounted retina. Retinal ischemia was induced in normal rats by raising the intraocular pressure of one eye to 100 mmHg for 60 min with saline injected by a peristaltic micro-pump, causing the eye to blanch. This technique causes ganglion cell apoptosis for several days following reperfusion (28–30). TUNEL staining in the ischemic retina was compared to that in the contralateral (non-ischemic) retina. The three negative controls were: (1) sections of apoptotic prostate from which the terminal transferase enzyme was omitted; (2) sections from a normal prostate stained with TUNEL-HRP; and (3) the contralateral (non-ischemic) retina also stained with TUNEL-HRP.

For the colocalization study, TUNEL-FITC was used to stain 10-µm cryostat sections simultaneously with a polyclonal antibody to von Willebrand factor (vWF; 1:500; Dako Corp., Carpinteria, CA). The TUNEL-FITC protocol was followed by an overnight incubation with anti-vWF 1:100 goat serum and 0.1% Triton in PBS. The anti-vWF was detected with a rhodamine-conjugated donkey anti-rabbit IgG (1:600; Jackson ImmunoResearch Laboratories, West Grove, PA).

Postmortem eye specimens. Human eyes from anonymous donors were obtained from the National Disease Research Interchange (Philadelphia, PA). The following selection criteria were applied: < 65 yr old, with either insulin-requiring diabetes or no diabetes, no life-support measures, and no chemotherapy. The donors were selected in pairs to be approximately age matched. The eyes were enucleated an average of 5.3±2.5 h after death, and preserved by immersion in normal buffered formalin an average of 11.0±4.7 h after death. The interval from death to fixation was much shorter than that found to cause false positive TUNEL staining in central nervous system tissue (31). The eyes remained in formalin at room temperature for 1–2 wk before the retinas were dissected into quadrants by making four tangential cuts along the nasal, temporal, dorsal and ventral axes. The retinas were then washed for 2 min in PBS and two changes of reverse osmosis purified water, to remove excess salt. Further cuts were made in the radial edge of each quadrant to enable flat mounting on microscope slides coated with 6-aminopropyltriethoxysilane. The specimens were allowed to contact with the microscope slide for 24 h at 4°C and were then stained immediately by the TUNEL-HRP protocol as described above.
Data collection and analysis. The percent weight change of rats was calculated as the difference between the weights at STZ injection and sacrifice, divided by the initial weight and multiplied by 100. The blood glucose and percent weight change data were analyzed by Mann-Whitney or Kruskal-Wallis nonparametric tests. Analysis of the morphometry data was by ANOVA or unpaired t-test, where appropriate.

The total number of TUNEL-HRP–positive cells was recorded for each retina in a masked fashion. Each retina was visually scanned with a high power (40×) objective in a serpentine manner to observe the entire retina. The number of TUNEL-HRP–positive cells was expressed per unit area, measured by tracing the outline of each retina viewed with a dissection microscope attached to the image analysis system described above. TUNEL-HRP–positive cell count data was log$_{10}$ transformed to compensate for unequal group variances before analysis of variance. Individual differences between groups were measured using the Newman-Keuls test.

Results

The retina degenerates during diabetes. To determine if atrophy and cell loss occur in the retina during diabetes, the relative thickness of the layers of the retina were measured in diabetic and age-matched control rats. Image analysis of serial sections of paraffin-embedded eyes from rats after 7.5 mo of STZ diabetes was used to measure changes in the thickness of the entire retina, the inner plexiform and nuclear layers, and to quantify the ganglion cell density. To standardize for bias introduced by skewed sectioning angles, all measurements were expressed relative to the thickness of the choroid in each section, which did not vary significantly with diabetes, averaging 101.5±3.7 µm in diabetic rats and 105.7±8.1 µm in controls (t-test, t = 0.47, P > 0.6). The diabetic rats had a significantly reduced weight (percent weight change was 59.5±9.29 in the diabetic group and 154.3±13.47 in controls, n = 6, P < 0.001) and elevated blood glucose (154.3±13.47 mg/dl in diabetic group and 65.3±4.73 mg/dl in controls, P < 0.001). The thickness of the inner plexiform layer was reduced by 22% of control and the inner nuclear layer was reduced by 14% of control, representing a significant reduction (t-test, $P < 0.001$ in both cases; Fig. 1 A). The total thickness of the retina was also reduced by 13% of control. This reduction was completely accounted for by the reduced thickness in the inner plexiform and nuclear layers; the combined thickness of the outer plexiform and nuclear layers did not change (t = 0.775, P > 0.4). The average ganglion cell density in the diabetic rats was 72.8±0.84/100 µm, while that of controls was 80.5±1.19/100 µm (t-test, P < 0.001; Fig. 1 B), a 10% reduction. This reduction in the number of ganglion cell bodies, combined with the overall reduction in the thickness of the inner plexiform layer, indicates that there is a significant ganglion cell loss in the retina after 7.5 months of STZ diabetes. Additionally, the reduced thickness of the inner nuclear layer could be accounted for by the death of neurons and Müller cells. These data demonstrate that retinal degeneration occurs in diabetes and suggest that neural cells die at an accelerated rate compared to age-matched controls.

Retinal cell death occurs by apoptosis during diabetes. Morphometric analysis of sections of long-term diabetic rat eyes suggests that there is an accelerated rate of death of retinal ganglion cells, neurons, and possibly glial cells. To characterize and quantify this cell death, and to determine the time course of the increase, the number of apoptotic nuclei were counted in retinas after 1, 3, 6 and 12 mo of STZ diabetes. Because apoptotic cells are sparsely distributed in other retinal degenerations (22, 23), we stained whole-mount retinas using the TUNEL-HRP method to identify nuclei containing fragmented DNA, a characteristic of cells undergoing apoptosis, and the total number of TUNEL-HRP–positive cells in each retina was expressed per 0.5 cm$^2$. This enabled an estimate of the number of apoptotic nuclei in the entire retina, and provided a superior sampling technique compared with TUNEL staining in histological sections.

![Figure 1. Atrophy of the rat retina after 7.5 months of diabetes.](Image)

Various morphological parameters were measured by image analysis in 5 high power microscope fields along the retina in 16 sections from each of 6 diabetic and 6 control rat eyes. (A) The average thickness of the inner plexiform layer (IPL) and inner nuclear layer (INL) were reduced in diabetic rats (striped bars, *P < 0.001), compared to controls (open bars). The combined thickness of the outer plexiform layer and outer nuclear layer (OPL + ONL) was not different from control, but the overall thickness of the retina (RET) was significantly reduced in the diabetic rats (P < 0.001). (B) There were significantly fewer retinal ganglion cells (RGC) in diabetic rats (striped bars) compared to controls (open bars, *P < 0.001).

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<td>27.0±13.45*</td>
<td>201.6±29.06*</td>
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Rats were injected with streptozotocin and housed alongside age-matched controls for 1, 3, 6, and 12 months. % Weight change and plasma glucose were measured as described under Methods. Glycosylated hemoglobin values were also measured in blood samples taken at sacrifice in the one and six month groups. The % weight change, plasma glucose and glycosylated hemoglobin values were significantly different from control in all diabetic groups. (*P < 0.05, †P < 0.005, Mann-Whitney test).
All diabetic rats gained significantly less weight than their respective age-matched control groups (Table I). The blood glucose values of the diabetic rats were significantly higher than control values at all four time points ($P < 0.05$). Glycosylated hemoglobin, which is a measure of chronic hyperglycemia, was also significantly higher than control values in the diabetic groups at both time points measured.

TUNEL-HRP–positive cells were observed in retinas of diabetic rats (Fig. 2), indicated by a brown precipitate staining nuclei. These cells were easily recognized against negligible background staining and appeared identical to TUNEL-HRP–positive cells in both the ischemic retinas and the ventral prostate from castrated rats. In many instances, TUNEL-HRP–positive nuclei had a typical apoptotic morphology including pyknosis and nuclear fragmentation (Fig. 2). The vasculature of the retina could also be visualized with normal light field optics and was further resolved by differential interference contrast. The vast majority (> 90%) of TUNEL-HRP–positive cells were distinct from observable vasculature. TUNEL-HRP–positive cells appeared occasionally in all parts of the retina, mostly as individual cells or as loosely associated groups (Fig. 3) at different depths in the whole mounts, noted by focusing through the tissue.

Diabetic rats had significantly more TUNEL-HRP–positive cells at all four time points than their respective age-matched controls (Fig. 4; 2-way ANOVA with post-hoc New-
Insulin reduces apoptosis in the retina. To determine the effect of insulin on diabetes-induced retinal cell death during the first month of diabetes, rats were given subcutaneous insulin implants 3 d after STZ injection, and killed 1 mo later. The retinas were whole-mounted and stained with TUNEL-HRP as described earlier. The uncontrolled diabetic group (no insulin implants) gained significantly less weight ($P < 0.001$) and had higher blood glucose values than the control group ($P < 0.0001$) (Table II). The insulin-treated group had slightly elevated plasma glucose levels compared with controls ($P < 0.05$). Soon after the onset of diabetes, the average weight of the diabetic group became significantly less than that of the control group, while the insulin-treated group gained weight at the same rate but was significantly different from control values, while that of the insulin-treated group was significantly different from both the other groups ($**P < 0.0001$).
The average blood glucose in the diabetic group was high from the onset of diabetes, but that of the insulin-treated group gradually reduced to control levels over a 12-d period (Fig. 6B). As the insulin-treated rats gained weight, the number of implants given to each was increased to deliver the appropriate dose of insulin to maintain normoglycemia. The increase in the number of TUNEL-HRP–positive cells occurring in untreated diabetic rat retinas was consistent with our earlier findings (Fig. 7; *P*, 0.01, compared to respective control group). The number of TUNEL-HRP–positive cells in the retinas of the insulin-treated rats was significantly less than in the diabetic group (*P*, 0.05), and not significantly greater than the control group. These data suggest that early insulin replacement reduces the number of apoptotic cells in the retina of the diabetic rat. The effect of insulin suggests that the increase in TUNEL-HRP reactivity is not due to a confounding effect of STZ injection, but is a direct consequence of diabetes.

Apoptosis is increased in the retinas of humans with diabetes. To determine if retinal apoptosis occurs to a similar degree in the retinas of humans with diabetes, we examined five postmortem human retinas from donors with and without diabetes. The retinas were divided into quadrants, flat-mounted, and labeled using the TUNEL-HRP protocol in the same way as the rat retinas in earlier experiments. Adhering retinal pigment epithelial cells were mostly washed off the specimens before flat-mounting, and the few remaining pigmented cells were easily identified by their position below the plane of the retina, and were not confused with TUNEL-HRP–positive cells. Table III summarizes the clinical details of each donor along with the number of apoptotic cells per cm². Subjects 1, 2, and 3 had no history of diabetes, while Subjects 4 and 5 had confirmed histories of diabetes for 6 and 30 yr, respectively, and both used insulin. Subject 5 also had a confirmed history of diabetic retinopathy. The vasculature of each retina was examined with both light field and differential interference contrast optics for indications of abnormalities. The retina of Subject 5 contained many microaneurysms typical of diabetic retinopathy. The retina of Subject 3 also contained an area
characteristic of branch vein occlusion, in which vasculature was not clearly visible. No other vascular abnormalities were observed in the other specimens. Many TUNEL-HRP–positive cells had typical pyknotic morphology and were mostly distributed as single cells (Fig. 2). The subjects without diabetes had an average TUNEL-HRP–positive cell count of 70.9±21.9 cells/cm² (58±14.8 cells/cm², excluding Subject 3), while the subjects with diabetes had an average of 145±20.8 cells/cm². These data indicate that retinal apoptosis is a generalized response of retinal neural cells to diabetes and is not a specific feature of STZ-induced diabetes in rats. Furthermore, TUNEL-HRP–positive cells were observed away from microaneurysms in a human retina with diabetic retinopathy, and are therefore not limited to areas with retinal vascular lesions.

**Discussion**

This study reports that both experimental diabetes in rats and diabetes mellitus in humans are accompanied by increased apoptosis of retinal neural cells. The increase in the frequency of apoptosis occurred after only 1 mo of experimental diabetes in rats, and a similar increase was noted in a human retina after six years of diabetes. The significant reduction of retinal ganglion cells and the reduction in the thickness of the inner plexiform and nuclear layers after 7.5 months of STZ diabetes suggest that the apoptotic cells are likely to include ganglion cells and other neurons. The histological location of TUNEL-positive cells and lack of colocalization with von Willebrand factor further suggest that neuronal and/or glial cell apoptosis occurs in the retina in diabetes. Viewed in the light of previous observations of neuronal cell death in diabetes (7–9), these data strongly suggest that neuronal cells begin to die soon after the onset of experimental diabetes in rats. They also confirm another brief observation of apoptosis in retinas of humans with diabetes, which were included in the control group of a study reporting apoptosis in open angle glaucoma (23). Therefore, neurodegeneration should be considered a component of diabetic retinopathy, as was suggested by both Wolter and Bloodworth (7, 8). Indeed, focal areas of retinal thinning have also been described in patients with diabetic retinopathy (32), and nerve fiber layer defects occur in patients with minimal or no vascular lesions (33).

Accelerated apoptosis of retinal vascular cells has been measured in trypsin-digested retinas from both rats and humans with diabetes (3), but the magnitude of apoptosis reported here indicates that most of the apoptotic cells in the retina are not endothelial cells or pericytes. Since quenching of endogenous peroxidase greatly reduced background staining associated with the vasculature and erythrocytes, TUNEL-HRP–positive cells were not confused with components of the blood. Furthermore, the increase in TUNEL-HRP staining is unlikely to be due to ischemic necrosis since it occurs after only one month of experimental diabetes in rats, before the onset of any known vascular occlusion. Neural cell death due to increased vascular permeability cannot be ruled out, however, since some increase in permeability can occur after one month of experimental diabetes in rats (34).

This study quantified the number of apoptotic cells in diabetic rat and human retinas by surveying whole-mount retinas. Other studies have observed some TUNEL-positive labeling in cryostat sections of diabetic rat eyes (35) and human retinas (23). Judging from the total number of apoptotic cells counted in the present study, it is unlikely that TUNEL staining of histological sections can be used to accurately quantify apoptosis in the diabetic retina, since a very large number of sections would have to be stained to make a reasonable sample. The section containing TUNEL-positive nuclei illustrated in Figure 5 was one of many radial sections that were found to contain no TUNEL-positive staining. This pattern of staining of radial sections is in line with the distribution of TUNEL-positive nuclei illustrated in Figure 3. It is clear that apoptosis also occurs in the normal retina, so in order to demonstrate an increase in apoptosis above baseline levels we sampled the entire retina.

Could the frequency of apoptosis explain the reduction in retinal thickness? The rat pup retina has 24.8±0.96 million cells at postnatal day 8, at which time the percentage of mitotic cells is negligible (36); so a reasonable estimate of the total number of retinal cells in the adult rats used here is ~20 million. Estimating the length of time that cells contain fragmented DNA as 6–12 h (37), the number of apoptotic cells measured here predicts a total loss of retinal cells of 1–10% over a 12 mo period. This is consistent with the morphometric changes we measured in the rat retinas after 7.5 months of diabetes. This degree of neural cell loss would not be detected clinically since the neural retina is normally transparent. The gradual nature of the cell death is consistent with the long latent period between the onset of diabetes and detectable diabetic retinopathy.

The increase in apoptotic cells in diabetic rats was mirrored in postmortem retinas from humans with diabetes. These data confirm that retinal neural cell death is not specific to STZ diabetes in rats. It is important to note that the number of apoptotic cells in both human retinas was about the same, but only one had detectable vascular lesions. The other donor was known to have had diabetes for only six years, and clinically evident vascular abnormalities do not usually develop during this short a duration of diabetes in humans. Therefore, these data suggest that retinal apoptosis is not a late consequence of vascular lesions.

Insulin delivered during the first month of diabetes reduced the number of apoptotic cells in the retina. These data suggest that retinal apoptosis in diabetes can be reversed by insulin therapy. Insulin may act by reversing some aspect of the physiology of diabetes (such as hyperglycemia). Indeed, galactosemic rats, which are hyperglycemic but not insulin deficient, also have increased vascular cell apoptosis (3). There is also extensive evidence that insulin can act as a trophic factor that affects cell survival in the central nervous system. Insulin reduces neuronal cell death in cerebral ischemia independently of blood glucose levels (38). Insulin regulates a number of factors associated with apoptosis, including neuronal intracellular calcium levels (39), calretinin and calbindin (40). Furthermore, insulin is a required survival factor in the central nervous system. At physiological concentrations, insulin rescues cultured optic nerve oligodendrocytes from death induced by trophic factor withdrawal (41), and is necessary for survival of retinal ganglion cells in culture (42). Insulin also rescues retinal vascular endothelial cells when apoptosis is induced by high glucose media (43). Regardless of the mechanism, these data strongly suggest that retinal apoptosis is reduced by exogenous insulin.

Secondary toxic factors may also contribute to retinal apoptosis in diabetes. Changes in glutamate metabolism occur in short-term experimental diabetes (6), implying that glial cells in the diabetic retina provide less protection from extracellular...
glutamate accumulation. An increase in the amount of unmetabolized glutamate in the retina may cause glutamate toxicity, which may in turn lead to neuronal apoptosis. Furthermore, retinal glial cells express more glial fibrillary acidic protein in early experimental diabetes (6) and in human diabetes (5), indicating a reactive state typical of central nervous system injury.

An increase in neural cell apoptosis could also contribute to microangiopathy because the apoptotic cells may include glia. Glial cells induce barrier properties in brain (44) and tight junction protein expression in retinal vascular endothelial cells (45). If retinal glial cells die, this could lead to a loss of endothelial cell barrier properties. Thus, retinal astrocyte or Müller cell death could impair barrier function. Indeed, other results from this laboratory show that the tight junction protein, occludin, is reduced in rat retinas after short-term STZ diabetes (Antonetti et al., manuscript submitted for publication).

This study addresses the two questions posed in the introduction. The data suggest that: (1) apoptosis begins soon after the onset of diabetes and continues at a relatively constant rate; and (2) the apoptotic cells include ganglion cells and other neural cells. Removal of insulin by STZ gives rise to neural cell death in the retina, but it is not yet established whether this relationship is a direct one or if the loss of insulin gives rise to apoptosis by an indirect mechanism, such as hyperglycemia. In light of these data, we suggest that diabetic retinopathy should be viewed as a disease that includes retinal neurodegeneration as well as vascular pathologies. Further attempts to understand and treat this disease must include a consideration of the roles of all three cellular elements (neurons, glial and vascular cells), and how they interact under the physiological constraints of diabetes.

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References


