2-DEOXY-D-GLUCOSE UPTAKE IN THE INNER RETINA: AN IN VIVO STUDY IN THE NORMAL RAT AND FOLLOWING PHOTORECEPTOR DEGENERATION

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ABSTRACT

Purpose: To evaluate, in vivo, at the cellular level, glucose metabolism in the rat inner retina, and to determine how inner retinal glucose metabolism is affected by photoreceptor degeneration.

Methods: Glucose metabolism was evaluated using the 2-deoxyglucose technique. This is an autoradiographic technique that permits evaluation of glucose uptake at the cellular level. The three experimental groups consisted of normal rats (n=13), dystrophic Royal College of Surgeons rats (n=3), and rats previously treated with argon green photocoagulation (n=5).

Results: Deoxyglucose uptake in the normal rat was not uniform across the inner retina. Uptake was greatest at the junction of the outer plexiform and inner nuclear layers, and in the inner plexiform layer. Following focal or diffuse photoreceptor loss, there was a marked decrease in the amount of deoxyglucose uptake at the junction of the outer plexiform and inner nuclear layers.

Conclusion: The pattern of uptake of deoxyglucose in the inner retina is consistent with abundant uptake of deoxyglucose by Müller cells and at sites of synaptic transmission. The decline in deoxyglucose uptake following diffuse or focal photoreceptor loss indicates that there is diminished inner retinal glucose uptake following photoreceptor loss. This change in inner retinal glucose metabolism following photoreceptor loss may help to explain the inner retinal vascular changes observed following photocoagulation and in retinal dystrophies.

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INTRODUCTION

BACKGROUND OF RETINAL ENERGY METABOLISM AND ITS MEASUREMENT

The retina, in common with other central nervous system tissue, requires a high level of energy metabolism. Energy is used in the retina for a host of cellular functions that are common to all cells, including maintenance of ion gradients across cell membranes, protein synthesis, and nucleic acid transcription. In addition, the retina has an increased energy metabolism burden to support the energy requirements of phototransduction and neurotransmission. These latter requirements are very energy-intensive functions and account for the extremely high level of energy consumption by the retina.[1,2] The retina depends principally on the metabolism of glucose as a source for energy, although oxidation of aspartic acid and glutamic acid may account for a portion of retinal oxygen consumption.[3-5]

Retinal energy metabolism has been the subject of intensive research. However, relatively few studies have been performed to correlate retinal energy metabolism and the cellular processes for which it is being used. This is because of the difficulty in measuring the rate of energy metabolism in small regions (eg, one cell) or in isolated units performing a specific cellular function (eg, synaptic transmission).

Most studies of retinal energy metabolism have concentrated on the role of oxygen in retinal energy metabolism rather than the role of glucose or other metabolic substrates. This is largely due to the greater ease of investigational approaches to assessing oxygen consumption and the more complex role of glucose in metabolic pathways not directly related to the production of adenosine triphosphate (ATP). However, several studies of glucose metabolism have been performed, and they are briefly summarized as follows.

In Vitro Studies of Glucose Metabolism

In vitro studies of retinal glucose metabolism have been performed in the rat,[3,5] the rabbit,[1,2,6] the guinea pig,[7,8] and the human.[9] In general, these studies have used isolated retinas maintained in perfusion chambers under very controlled conditions. The studies in the guinea pig and the human are an exception to this generality in that some of the studies involved investigations of isolated Müller

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cells[8,9] or Müller cell photoreceptor complexes.[8] These studies have been very enlightening with regard to glucose metabolism in the retina. They have raised several interesting questions that form the basis for the hypothesis to be evaluated in this thesis.

Ames and coworkers,[1] working in the rabbit, found that the energy for neurotransmission through the inner retina was obtained from the glycolytic metabolism of glucose. This finding was based on the abolition of the b wave of the electroretinogram and a decrease in lactate production with relatively little change in oxygen consumption when neurotransmission was blocked with specific inhibitors. These investigators found that inhibitors of neurotransmission decreased the rate of glycolysis in the dark-adapted inner retina by 79% ± 9%. Similarly, increased neurotransmission through the inner retina caused by a flashing light in the absence of inhibitors increased glycolysis by 48% ± 4%. These findings were consistent with those of Lowry and coworkers,[10] who found that the inner retina of the rabbit contained almost exclusively glycolytic enzymes in its inner layers. The findings by the investigators in the Ames group led them to suggest that there may be an obligatory dependence of neurotransmission on glycolytic, rather than oxidative, energy.[1] This would be consistent with the specific dependence of other functions in smooth muscle[11] and the central nervous system[12-16] on glycolytic energy.

Winkler[5] performed a comprehensive, quantitative study of glucose metabolism in vitro in normal and dystrophic rats. He found that 90% of glucose utilized aerobically by both normal and dystrophic rats was used in glycolysis. This finding suggested that aerobic glycolysis is the major pathway for glucose breakdown in all retina cell layers. Winkler also found that there were only small light-dark differences in retinal glycolysis and oxygen consumption. In contrast, oxidation of glucose was diminished in the light, compared with darkness, by 25% in the whole retina and by 40% in the photoreceptor cells. This suggests that light leaves unchanged or increases the amount of glucose metabolized by oxidation by the inner retina.

Poitry-Yamate and coworkers,[8] working with Müller cells and Müller cell photoreceptor complexes isolated from guinea pig retinas, found that glucose taken up by Müller cells was metabolized to carbohydrate intermediates and that a large amount of lactate was released into the tissue bath by the isolated Müller cells. Furthermore, these investigators showed that the lactate in the tissue bath could be taken up and used as a metabolic substrate for oxidative metabolism by photoreceptors. In fact, they found that lactate was preferred over glucose by the photoreceptors. These findings led the investigators to suggest that, in vivo, the Müller cell transfers lactate to the photoreceptors. This is consistent with the same group's studies using the 2-deoxyglucose technique (see below) in vitro to evaluate glucose metabolism in the whole retinas isolated from guinea pig[7] and in vivo in the honeybee.[17] In those studies, these investigators found that glucose uptake was predominantly in glial cells, leading them to suggest that some metabolite must be transferred to the photoreceptors from the glial cells to satisfy the large need of ATP in the photoreceptors.

Winkler and coworkers,[9] working with cultured human Müller cells, found that glycolysis accounted for 99% of the glucose consumed by these Müller cells. Oxidative metabolism of glucose by mitochondria accounted for only 1% of glucose consumed.

There have been three other in vitro studies of glucose metabolism using the 2-deoxyglucose technique. These were done in the frog,[18] goldfish,[19] and monkey retinas.[20] Interestingly, these studies showed relatively greater uptake of deoxyglucose over photoreceptors, in contrast to the findings of predominantly glial cell uptake reported by Poitry-Yamate in the guinea pig.[7]

In Vivo Studies of Glucose Metabolism

Bill and coworkers have performed numerous studies of glucose metabolism using either the 2-deoxyglucose technique or direct measurements of glucose consumption by measuring arteriovenous differences in blood glucose concentrations. Their studies have been performed in the rabbit,[21] the cat,[22] the pig,[23,24] and the monkey.[25-27]

To briefly summarize their studies, they have concluded that in the inner retina of the pig, in the dark, 69% of the glucose is consumed in oxidative metabolism and 21% in glycolysis. In the outer retina of the pig, in the dark, 61% of the glucose is consumed in glycolytic metabolism, while 12% is consumed in oxidative metabolism. In the rabbit, both in light and dark, these investigators found that for the whole retina, 10% of glucose was consumed in oxidative metabolism and 50% in glycolysis. (The balance of the glucose utilization in all of these studies was presumed to be in other processes, such as protein synthesis).

Vitreoperfusion following cessation of retinal and choroidal blood flow after death has been used to measure total glucose consumption by the retina in cats.[28] This modality has the advantage of perhaps being suitable to the study of glucose metabolism in humans undergoing vitrectomy and allowing the simultaneous measurement of oxygen consumption.[29]

The extensive information provided by these studies raises some very interesting questions with regard to glucose metabolism in pathologic states. In diabetic retinopathy, it has long been observed that destruction of photoreceptors with photocoagulation causes a reduction in neovascularization of the inner retina. Similarly, atten-

uation of retinal vessels is a well-known occurrence in the retinal dystrophies with extensive photoreceptor atrophy. Experimentally, Wilson and coworkers[30] have demonstrated that destruction of photoreceptors leads to a delayed loss of inner retinal vessels in the monkey. The changes in the inner retinal vessels following loss of photoreceptors has generally been attributed to an increase in inner retinal oxygen tension. However, the studies described above raise two other possible explanations for the attenuation of the retinal vessels following destruction of photoreceptors: (1) Since Ames and coworkers[1] found that neurotransmission through the inner retina was responsible for a large percentage of the inner retinal energy metabolism, it is possible that following photocoagulation, there could be less of an energy requirement in the inner retina because of decreased neurotransmission through the inner retina. (2) The observation of Poitry-Yamate[8] that there appears to be transfer of lactate from Müller cells to photoreceptors indicates that there is a link between the metabolism of the inner retina (Müller cell) and the outer retina. In the absence of photoreceptors, one would anticipate that there would be less glucose metabolism by Müller cells. The present study was undertaken to evaluate how neurotransmission and photoreceptor atrophy affect inner retinal glucose metabolism.

HYPOTHESIS

There is diminished use of glucose in the inner retina following photocoagulation and in retinal dystrophies that result in loss of photoreceptors. This decrease in glucose utilization is due to diminished neurotransmission through the inner retina and decreased glucose consumption by Müller cells.

MATERIALS AND METHODS

CHOICE OF TECHNIQUE

In 1976 and 1977 Sokoloff and coworkers[31,32] developed a technique for the in vivo or in vitro measurement of energy metabolism called the deoxyglucose technique. This technique utilizes a radioactively labeled glucose analogue to serve as a marker of glucose metabolism. In the central nervous system and in the retina, the metabolism of glucose is utilized as the principal energy source.[3,5] Because of the use of glucose as an energy source, the deoxyglucose technique can be utilized to study energy metabolism in the central nervous system and retina. This technique has been of great value in the study of the central nervous system's regional differences in energy metabolism and variation in energy metabolism in different physiologic and pathologic states.[12] The principles of the deoxyglucose technique form the basis for positron emission tomography (PET) scanning, which is now used as a clinical diagnostic tool for central nervous system disease. The deoxyglucose technique has been used less in the study of retinal physiology and pathology.

This technique is attractive because, unlike studies that evaluate glucose consumption using arteriovenous differences in glucose concentration, the deoxyglucose has the advantage of showing glucose uptake at the cellular level. This technique remains the only method to evaluate the relative glucose utilization of specific cells within a tissue and seems ideally suited to answer the questions of how photoreceptor degeneration and photocoagulation affect Müller cell glucose utilization. Deoxyglucose is available commercially, radioactively labeled with either ^{14}C or ^{3}H. To achieve the greatest resolution of individual cell glucose uptake, it is preferable to use deoxyglucose labeled with ^{3}H.

The deoxyglucose method may be used in a qualitative or quantitative fashion. In order to measure glucose consumption quantitatively, one must measure the entire history of the plasma deoxyglucose concentration over the course of the experiment, the steady state of the plasma glucose concentration, and the local tissue concentration of deoxyglucose. In this initial study, I have chosen to use this technique in a qualitative fashion to evaluate the local distribution of deoxyglucose. Qualitative interpretation of the autoradiograms allows one to make relative statements regarding the glucose metabolism of adjacent cells and tissues, but it does not allow one to place a numerical value on glucose utilization.

The disadvantages of the technique are that it is complex and expensive. In addition, glucose metabolism is complex, and interpretation of measurements of glucose uptake is affected by many variables. Chief among these is that tissues may metabolize glucose through either glycolysis or the tricarboxylic acid cycle. The route of metabolism of glucose affects the amount of glucose uptake by a particular tissue, as glycolysis is a much less efficient pathway for the generation of ATP than is the tricarboxylic acid cycle. Furthermore, there are other metabolic fates for glucose than the generation of ATP, and these must be kept in mind in evaluating the results of deoxyglucose studies.

CHOICE OF EXPERIMENTAL ANIMAL

As detailed in the introduction, the findings of previous studies have yielded different results for the glucose metabolism of the inner retina.[1,5,6,23] For example, Bill and coworkers found a much lower percentage of glucose consumed by glycolysis in the inner retina of the pig[23] than Winkler found in the inner retina of the rat.[5] These different results could be due to different experimental approaches or to species differences. I have chosen to evaluate retinal energy metabolism in the rat. To evaluate

the in vitro observations of Poitry-Yamate[7,8] and Ames[1] discussed above, it is preferable to work in vivo. Use of the 2-deoxyglucose technique to evaluate glucose metabolism at the cellular level requires working in a small animal; working in a larger animal would be prohibitively expensive because of the amount of radioactive material necessary. Furthermore, much is known about the retinal energy metabolism of the rat from previous in vitro studies of glucose[3,5,33-36] and oxygen[37-39] metabolism in this animal.

BIOCHEMICAL THEORY

The deoxyglucose method for the measurement of local glucose utilization was developed by Sokoloff.[31,32] 2-deoxy-D-glucose (2-DG) differs from glucose in that a hydroxyl group on the second carbon atom has been replaced by a hydrogen atom. 2-DG and glucose are transported between blood and brain tissues by the same saturable carrier. In tissues, deoxyglucose competes with glucose for hexokinase, which phosphorylates both to their respective hexose-6-phosphates. However, since 2-deoxy-D-glucose-6-phosphate (2-DG-6-P) lacks a hydroxyl group at the second carbon atom, it cannot be isomerized to fructose-6-phosphate by phosphohexoseisomerase. Consequently, 2-DG-6-P does not proceed farther down the glycolytic pathway. Since 2-DG-6-P does not appear to be a substrate for glucose-6-phosphate dehydrogenase, and since the brain and retina[34] have very little deoxyglucose-6-phosphatase activity, 2-DG-6-P accumulates within cells in these tissues. When radioactively labeled, 2-DG can therefore serve as a quantitative or qualitative marker of glucose utilization.

As with other methods of evaluating retinal energy metabolism, the deoxyglucose method also has its limitations. The use of this method in a quantitative fashion requires that deoxyglucose be present in trace amounts, that the arterial plasma glucose concentration remain constant, and that the glucose metabolism of the retina be maintained in a steady state. Also, it is important that 2-DG-6-P remain in the cell and not be converted to other substances. The retina contains low levels of glucose-6-phosphatase,[34] making it unlikely that 2-DG-6-P is converted back to 2-DG. However, another concern is that 2-DG-6-P might diffuse out of cells after fixation. Investigators have shown that 2-DG-6-P is trapped within cells prior to fixation, but that there is some ability of 2-DG-6-P to diffuse out of cells after fixation.[18] Therefore, to prevent diffusion of 2-DG-6-P, tissue needs to be processed to minimize exposure to aqueous solutions. Another concern is that the radioactivity initially present on deoxyglucose ends up associated with glycogen. One report has measured that as much as 30% of the radioactivity initially associated with deoxyglucose migrated with glycogen following incubation with retinal tissue.[18]

Finally, it is important to note that the deoxyglucose technique is a measure of glucose utilization. The uptake of 2-DG will be influenced by whether a cell or cell function is coupled with the glycolytic metabolism of glucose as opposed to the oxidative metabolism of glucose. The deoxyglucose technique would be expected to measure a much larger glucose uptake in cells or cell processes that are generating energy through the glycolytic metabolism of glucose, as this is much less efficient at generating ATP than is oxidative metabolism. Glycolytic metabolism of glucose generates only 1 molecule of ATP for every 17 or 18 molecules of ATP generated by the oxidative metabolism of glucose.[16] Consequently, cells relying primarily on glycolytic metabolism of glucose will be more heavily labeled on autoradiograms using the deoxyglucose technique.

ANIMALS

Adult male Long-Evans, Sprague-Dawley, and Royal College of Surgeons (RCS) rats were used in this study. The animals were housed in the Oregon Health Sciences University Animal Care Facility and were treated within the standards set by the Animal Care Committee at that institution. These standards conform to the guidelines established by the Association for Research in Vision and Ophthalmology. The animals received food and water ad libitum. They were kept at a 12-hour light, 12-hour dark-light cycle. All procedures were carried out in room light.

Controls

Healthy Long-Evans (n= 4), Sprague-Dawley (n= 8), and RCS nondystrophic (n=1) rats were studied with the deoxyglucose technique to determine the normal retinal distribution of deoxyglucose. In addition, the fellow eye of each of the animals treated with photocoagulation served as a control in those animals.

Photocoagulation

After anesthesia was obtained with a cocktail (1 mL/kg body weight) of acepromazine maleate (1 mg/mL), xylazine hydrochloride (5 mg/mL), and ketamine (50 mg/mL), five Long-Evans rats were treated with argon green photocoagulation. The photocoagulation burns were placed in a sparse pattern around the optic nerve, sparing the area centralis. The intensity of the burns was adjusted to obtain a light white burn, similar to the photocoagulation burns used to treat macular edema.

Dystrophic RCS Rats

Dystrophic RCS rats (n=3) were studied with the deoxyglucose technique 24, 38, and 48 days after birth.

DEOXYGLUCOSE TECHNIQUE

After anesthesia was obtained with a cocktail (see above)

of acepromazine, xylazine, and ketamine, each rat was injected with 25 mCi of ^{3}H-2-deoxy-D-glucose reconstituted in 1 mL of sterile normal saline via the saphenous vein. The animal was then allowed to recover from the anesthetic. At 90 minutes after the deoxyglucose injection, the rat was anesthetized in an ether chamber and sacrificed by decapitation.

The eyes were immediately enucleated and snap frozen in Freon 22 chilled to -175°C with liquid nitrogen. The eyes were then "freeze substituted" by placing them into prechilled anhydrous ether (-70°C) containing water-extracting molecular sieves. The eyes were kept at -70°C for 72 hours, then at -4°C for 24 hours, and then at 12°C for 24 hours. At room temperature the freeze-substituted eyes were fixed in 1% OsO_4 in acetone. The eyes were then gradually infiltrated with epon-araldite. After polymerization, 2-µm-thick sections were cut on a Reichert-Jung ultramicrotome. The sections were coated with NTB-2. Autoradiograms were developed at various time intervals, but exposures of 6 to 12 weeks proved to be best.

BIOCHEMICAL FATE OF DEOXYGLUCOSE

The biochemical fate of deoxyglucose was analyzed using a specific extraction for glycogen and thin-layer chromatography. One rat was treated as described above, but after enucleation the retinas from both eyes were quickly dissected free of the other tissues, frozen in liquid nitrogen, and lyophilized. The lyophilized retinas (3 mg dry weight) were homogenized in 300 µL of 1M $HClO_4$; 10 µL was saved to determine the radioactivity of the homogenate.

To determine how much deoxyglucose was incorporated into glycogen, a specific glycogen extraction was performed on a 50-µL aliquot of the retinal homogenate as described by Evequoz.[40] Briefly, the 50-µL aliquot of retinal homogenate was combined with 50 µL of HCL. This 100-µL sample was applied to Whatman 3M chromatography paper, washed three times for 40 minutes in 66% ethyl alcohol, rinsed briefly in acetone, dried, and counted in a scintillation counter.

Thin-layer chromatography (TLC) was used to determine the relative concentrations of 2-DG and 2-DG-6-P. A total of 240 µL of retinal homogenate was added to a 7.5% aqueous solution of $Ba(OH)_2$ and a 5% aqueous solution of $ZnSO_4$ $7H_2O$; 34 µL of NH_4OH was added to neutralize the perchloric acid. After vortexing, the mixture was centrifuged for 20 minutes. The pellet and an aliquot of the supernatant were saved for radioactivity counting. The remaining supernatant was lyophilized and reconstituted in distilled water. 20 µL of reconstituted supernatant and 10 µL of cold carrier (containing 50 µg of 2-DG and 50 µg of 2-DG-6-P) were placed on cellulose/plastic sheets (Merck 5577) with a solvent system consisting of ethylacetate: acetic acid: pyridine: H_2O (5:1.7:2.5:2.5). The plates were sprayed with a mixture of 0.5 g carbazols, 95 mL of ethyl alcohol, and 5 mL of H_2SO_4 and developed for 10 minutes at 120°C. Spots corresponding to 2-DG and 2-DG-6-P were scraped from the TLC plates and counted.

EFFECT OF AQUEOUS EXPOSURE ON DEOXYGLUCOSE LOCALIZATION

Some investigators[7,41] have stressed the importance of eliminating aqueous exposure of 2-DG–containing tissues. Prior to fixation, 2-DG can freely diffuse out of cells, while 2-DG-6-P remains trapped within cells, even in aqueous environments. Following fixation, 2-DG-6-P may also diffuse out of cells. However, superior tissue morphology can be obtained on thick sections briefly exposed to water during slide preparation and application of photographic emulsion. The amount of radioactivity lost in aqueous solutions after plastic embedding has not been previously evaluated. To quantitate potential loss of radioactivity, we analyzed the loss of radioactivity in the following extreme conditions: 200 1-µm plastic sections of rat retina were heated in 1 mL of distilled H_2O at 40°C for 20 minutes. The sections were then removed and the H_2O was added to 1 mL of a saturated solution of potassium hydroxide in methanol, acetone, and benzene. The amount of radioactivity present was determined in a scintillation counter. The total amount of radioactivity in 200 1-µm-thick sections of rat retina was determined by dissolving 200 sections in 1 mL of a saturated solution of potassium hydroxide in methanol, acetone, and benzene. One milliliter of water was added, and this solution was also counted in a scintillation counter.

HISTOPATHOLOGIC TECHNIQUES

The following standard and immunohistochemical stains were prepared on sections of rat retina to allow correlation with the autoradiograms: hematoxylin-eosin, periodic acid Schiff, Feulgen (for glycogen), vimentin (Müller cells), and synaptophysin.

RESULTS

BIOCHEMICAL FATE OF DEOXYGLUCOSE

Only 0.31% of the retinal radioactivity was present in glycogen. The remainder of the retinal radioactivity was present as 2-DG and 2-DG-6-P. The ratio of 2-DG-6-P to 2-DG was 3:1.

EFFECT OF AQUEOUS EXPOSURE ON DEOXYGLUCOSE LOCALIZATION

Eight percent of the total radioactivity was lost into warm water after heating at 40°C for 20 minutes.

DEOXYGLUCOSE TECHNIQUE

Following freeze substitution, the retinal morphology was sufficient to allow detailed identification of the retinal layers. Certain artifacts of freezing were present but did not interfere with the interpretation of the results. Freezing results in the formation of water crystals in the tissue. Because of the rapid freezing employed in this study, the water crystals were small but still evident in some preparations. In addition, freeze substitution created a dry tissue that was prone to cracking. Again, this artifact was easily identifiable and did not interfere with interpretation of the results of the study (Figure 1). [Note: Staining of autoradiograms requires a compromise between greater visibility of the exposed grains in the photographic emulsion in an unstained section and greater visibility of the details of the retinal cells in a more darkly stained section. When necessary, unstained and variably stained sections have been provided to maximize the illustration of critical points.]

Radioactive deoxyglucose was clearly evident in various ocular tissues to a much higher level than background. This was evident in the cornea, with prominent labeling of the keratocytes and corneal endothelium, as well as in extraocular muscle. These internal controls helped to confirm that the labeling seen on autoradiograms reflected the location of deoxyglucose and deoxyglucose-6-phosphate and had not been significantly affected by diffusion of these substances in the course of processing (Figures 2 and 3).

DEOXYGLUCOSE UPTAKE IN CONTROL RETINAS

The pattern of deoxyglucose uptake was the same in the three different strains of rat used in this study. There was intense uptake of radioactivity in the outer plexiform, inner nuclear, and inner plexiform layers. The pattern of uptake in the outer plexiform layer was such that there were focal areas of intense uptake at the junction of the outer plexiform layer and the inner nuclear layer. This intense uptake extended into the outer portion of the inner nuclear layer. These foci of intense uptake stood out in juxtaposition to adjacent areas that had markedly less uptake of deoxyglucose. The intense areas of radioactivity in the inner nuclear layer corresponded to cells in the outer portion of the inner nuclear layer, sometimes with adjacent cells taking up almost no deoxyglucose. The intense areas of radioactivity in the outer plexiform layer appeared to correspond to the expected position of synapses between the photoreceptors and cells of the inner nuclear layer, but these synapses were not visible at the light microscopic level.

There were prominent but slightly less intense areas of radioactive labeling within the inner plexiform layer and at the junction of the inner plexiform layer and the ganglion cell layer. In the inner plexiform layer, this labeling created two faint bands that ran parallel to the internal limiting membrane. There were also focal areas of intense radioactive labeling at the junction of the inner plexiform and ganglion cell layers. Notably, there was no labeling at the inner limiting membrane, at the junction of the Müller cells to their basement membrane (Figure 4A and B).

DEOXYGLUCOSE UPTAKE IN DYSTROPHIC RETINAS

At 24 days, dystrophic retinas showed markedly disarranged outer segments, but the outer nuclear layer was of normal thickness. By 38 days, there was moderate loss of the outer nuclear layer so that it was only one-half the thickness of the control RCS outer nuclear layer. There was continued disarrangement of the outer segments. By 48 days, there was marked atrophy of the outer nuclear layer so that it was only one-quarter the thickness of the control outer nuclear layer. Once again, there was marked disarrangement of the outer segments (Figure 5A and B).

The pattern of uptake in the dystrophic RCS rats was markedly different than in the control RCS animal. At the earliest time point examined (24 days), there was loss of the intense labeling at the junction of the outer plexiform and inner nuclear layers. There was relative preservation of the two less intense bands of labeling within the inner plexiform layer (Figure 6A, B, and C).

These changes were even more evident at the 48-day time point, at which there was markedly reduced deoxyglucose uptake in the region of the junction of the outer plexiform and outer nuclear layers. There was relative preservation of the two bands of deoxyglucose uptake in the inner plexiform layer. At this time point, there was striking uptake of deoxyglucose within some of the ganglion cells. There was also prominent deoxyglucose uptake within cell nuclei within the layer of disarranged outer segments (Figure 7A, B, C).

DEOXYGLUCOSE UPTAKE FOLLOWING PHOTOCOAGULATION

Photocoagulation burns resulted in focal areas of loss of the retinal pigment epithelium, the photoreceptor outer segments, and a variable number of the photoreceptor cell nuclei. There was some migration of pigment-containing cells into the outer plexiform and inner nuclear layers.

Deoxyglucose uptake in the area of photocoagulation was similar to that seen in photoreceptor degeneration in the RCS rat. There was loss of the intense deoxyglucose uptake at the junction of the outer plexiform layer and the inner nuclear layer. The labeling of the inner plexiform layer was relatively unaffected by the laser photocoagulation (Figure 8A, B, and C).

IMMUNOHISTOCHEMICAL AND HISTOCHEMICAL STAINS

Immunohistochemical stains for vimentin demarcated the Müller cells. Immunohistochemical stains for synaptophysin revealed prominent labeling in the inner and outer plexiform layers (Figure 9A and B).

DISCUSSION

This is the first in vivo study using ^{3}H-2-DG combined with freeze substitution to localize glucose uptake at the cellular level, in the mammalian retina. As would be expected, there is a diffuse background level of glucose utilization throughout the retina. However, as described in the "Results" section, there are distinct areas of greater glucose uptake. Analysis for diffusion of 2-DG and 2-DG-6-P indicated that only 8% of 2-DG and 2-DG-6-P were lost under extreme conditions. Similarly, only 0.31% of the retinal radioactivity was present in glycogen. These findings, coupled with the internal control findings of well-defined uptake of 2-DG in the corneal endothelium and extraocular muscle, make it unlikely that the pattern of 2-DG uptake seen in the retina is artifactitious.

The in vitro studies of Poitry-Yamate[7,8] and Ames[1] would suggest that there is increased glucose uptake in Müller cells and at sites of synaptic transmission. In order to compare the distribution of synapses and Müller cells to the pattern of 2-DG labeling, the retina was stained with an immunoperoxidase technique for vimentin and for syntaptophysin. Vimentin is present in Müller cells and synaptophysin is a glycoprotein that is present in the presynaptic vesicles of neurons.

I would interpret the pattern of uptake of 2-DG to be consistent with a high level of uptake by Müller cells and at sites of synaptic transmission. Interestingly, the pattern of 2-DG uptake does not correspond to the entire extent of the Müller cell. Specifically, the Müller cell endfeet do not show the extent of 2-DG uptake that is present over the remainder of the cell. This could indicate that there is some intracellular compartmentalization of glucose metabolism within the Müller cell. Such compartmentalization has been reported in the central nervous system.[14] Alternatively, it could be that 2-DG was lost from this site on account of diffusion, but this seems unlikely because diffusion of 2-DG from other ocular tissues (corneal endothelium and extraocular muscle) was quite limited.

The pattern of 2-DG uptake in this study is similar to that reported by Poitry-Yamate and associates working in the guinea pig.[7] Those investigators found prominent uptake of 2-DG within Müller cells of guinea pig retinas studied in vitro. Our results differ in that in Poitry-Yamate's study there was prominent uptake of 2-DG within the Müller cell endfeet. Also, in that study there was less uptake in the outer plexiform and inner plexiform layers than was seen in the current study.

The finding of a large amount of 2-DG uptake by Müller cells in this study is consistent with the in vitro studies of Poitry-Yamate and associates[8] and Winkler and colleagues[9] on isolated Müller cells from guinea pig and human, respectively. Those investigators have shown that there is a high rate of aerobic glycolysis in the Müller cell. Winkler concluded that the Müller cell utilized close to 99% of its glucose in glycolysis. The main products of interest from glycolysis are pyruvate (subsequently converted to lactate) and ATP. Poitry-Yamate[8] has shown that lactate produced by Müller cells is taken up by photoreceptors as an energy source. The ATP produced through glycolysis is used by the Müller cell for its many cellular functions, but a large portion of this ATP is undoubtedly consumed by the cells' role in maintaining ionic balances following neuronal cell depolarizations. One would expect that following loss of the photoreceptors, Müller cell glucose uptake to support photoreceptor metabolism, as well as the need for ATP for supporting ionic balance, would be reduced.

In the RCS retinas, there was loss of the pattern of deoxyglucose seen in the RCS control eye. By 24 days, even though the inner retina was intact, the distinct deoxyglucose uptake at the junction of the outer plexiform layer and the inner nuclear layer was diminished. There was some preservation of the bandlike labeling of the inner plexiform layer. By 48 days, there was even more extensive loss of 2-DG uptake at the junction of the outer plexiform and inner nuclear layers.

This change in the pattern of labeling is consistent with the observations of Poitry-Yamate and associates.[7,8] As already noted, these investigators have suggested that Müller cells metabolize glucose to lactate and that photoreceptors utilize the lactate as an energy source. In the RCS rat with degeneration of the photoreceptors, there is less deoxyglucose uptake by Müller cells, as there is less demand for lactate by the photoreceptors. Interestingly, the decrease in deoxyglucose uptake by the Müller cells occurs prior to the complete loss of the photoreceptors. In fact, the decrease in deoxyglucose uptake by Müller cells is readily apparent at day 24, when the cell bodies of the photoreceptors are still intact.

Similar findings are evident following photocoagulation. With local loss of the photoreceptors, there is loss of the intense deoxyglucose uptake at the junction of the outer plexiform layer and the inner nuclear layer. Once again, this is consistent with less uptake of glucose by the Müller cells as a result of the loss of photoreceptors.

In addition to deoxyglucose uptake in the Müller cells, there was clearly a fairly high level of deoxyglucose uptake in the outer plexiform layer and the inner nuclear layer that could not be accounted for by Müller cell

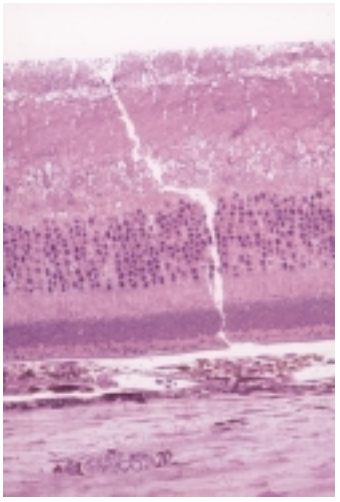

FIGURE 1
Histologic section of full-thickness retina, retinal pigment epithelium, choroid, and partial-thickness sclera. In center of figure is a crack resulting from drying of the tissue during freeze substitution and subsequent embedding. There are also small clear spaces due to formation of intracellular ice crystals. These are most evident in the nerve fiber and ganglion cell layers (toluidine blue, x400).

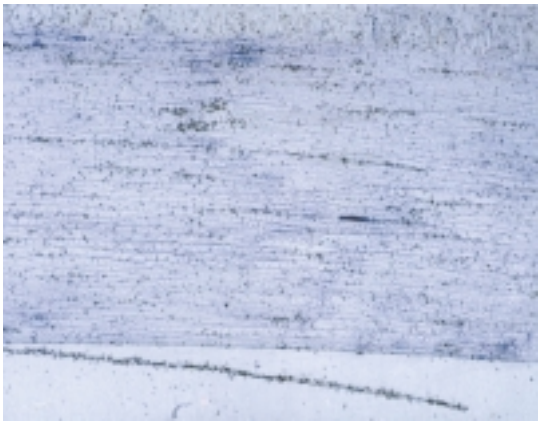

FIGURE 3
Autoradiogram of cornea with artifactitious separation of corneal endothelium. 2-DG uptake by corneal endothelium is sharply demarcated from background, indicating limited diffusion of 2-DG or 2-DG-6-P out of cells (toluidine blue, x200).

uptake alone. My interpretation of this relatively high level of uptake is that it is due to the use of glycolytic metabolism to support synaptic transmission at these sites. As already mentioned, there is some evidence in the central nervous system[12-16] and in smooth muscle[11] that glycolytic metabolism may be used to support specific functions on demand. There are large synapses present at the junction of the outer plexiform layer and the inner nuclear layer, a site of particularly high deoxyglucose uptake, as well as numerous synapses present in the inner plexiform layer. The labeling of the rat retina with synaptophysin roughly correlates with the non–Müller cell uptake of deoxyglucose. However, the resolution of the deoxyglucose

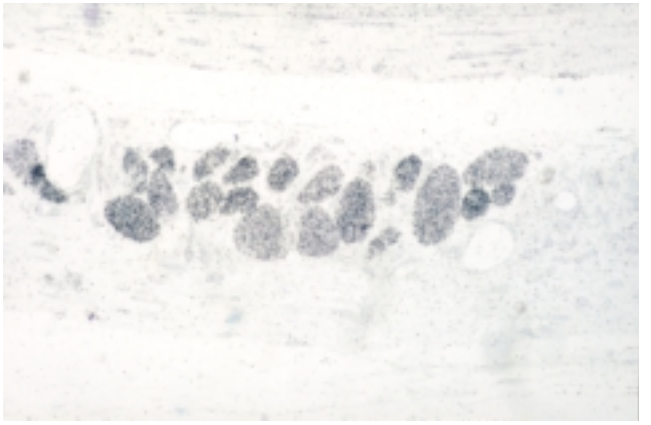

FIGURE 2
Autoradiogram showing 2-DG uptake by extraocular muscle relative to background. Labeling of the muscle is sharply defined relative to background. Slide is lightly stained to preserve visibility of grains of photographic emulsion (toluidine blue, x400).

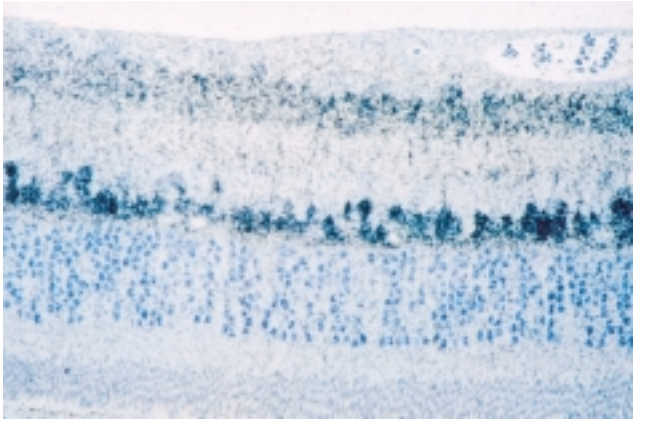

FIGURE 4A
Normal retina; RCS, nondystrophic. There is intense 2-DG uptake at junction of outer plexiform and inner nuclear layers, and in inner plexiform layer (toluidine blue, x400).

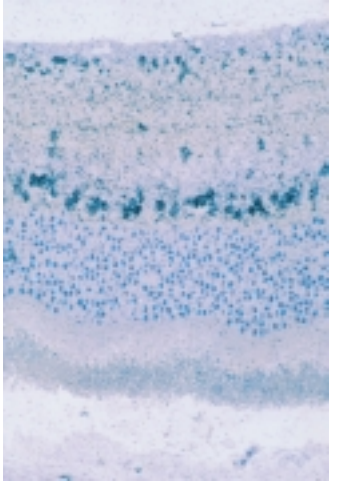

FIGURE 4B
Normal retina; Sprague-Dowley. Pattern of 2-DG uptake is similar to that shown in 4A, but the two faint bands of 2-DG uptake in inner plexiform layer are more prominent (toluidine blue, x400).

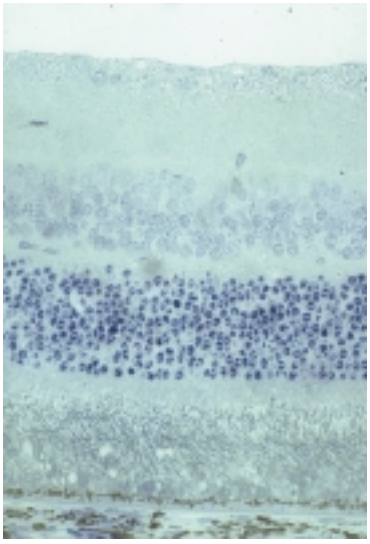

FIGURE 5A
24-day RCS rat retina. There is marked disarrangement of outer segments. Outer nuclear layer is of normal thickness (toluidine blue, x400).

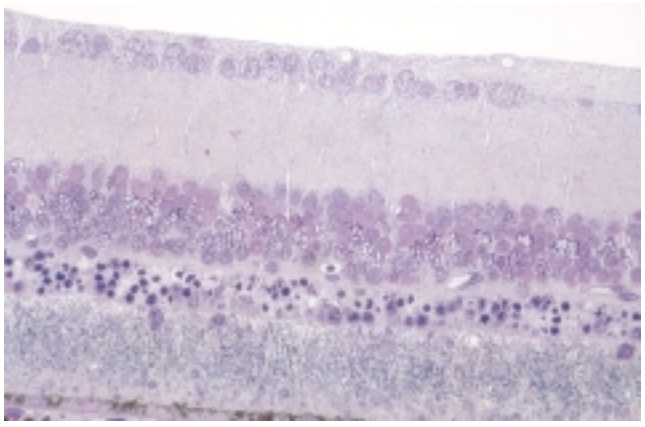

FIGURE 5B
48-day RCS rat retina. There is prominent atrophy of outer nuclear layer, with continued disarrangement of outer segments (toluidine blue, x400).

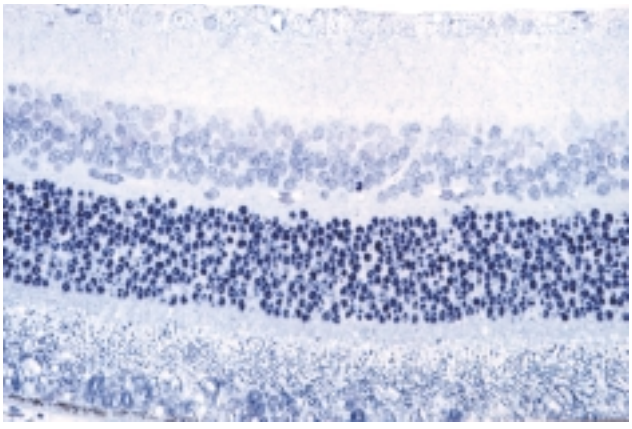

FIGURE 6A
24-day RCS rat retina. Histology for comparison to autoradiograms (toluidine blue, x400).

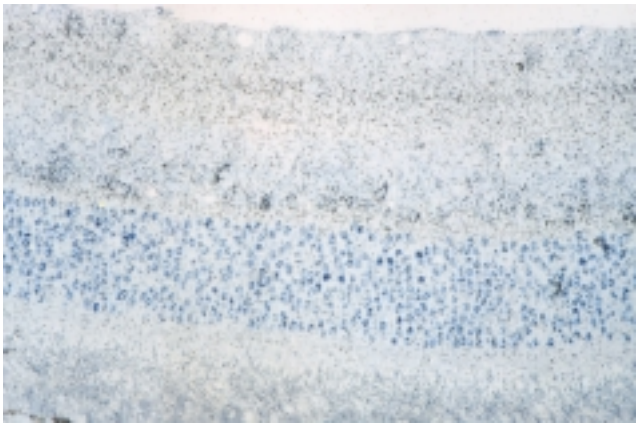

FIGURE 6B
24-day RCS rat retina. Lightly stained autoradiogram showing much less 2-DG uptake at junction of outer plexiform and inner nuclear layers compared to control. (See Figure 4 for comparison (toluidine blue, x400).

FIGURE 6C
24-day RCS rat retina. Unstained autoradiogram. Bands of 2-DG uptake are present in inner plexiform layer (x400).

localization in this study and the inadequate visualization of synapses in this light microscopic study do not permit a definite conclusion on this point. Further studies at the ultrastructural level with inhibitors of synaptic transmission might further clarify this issue.

It is more difficult to compare the results of this study to those of Bill and coworkers,[25-27] who studied retinal glucose uptake in the monkey using ^{14}C-2-DG. They reported that in the dark there was greater 2-DG uptake in the outer retina, as compared to the inner retina. In the light there was approximately equal uptake in the inner and the outer retina. The 2-DG uptake by individual cells was not reported in these studies.

It is of interest to try to correlate the findings in this study with studies of oxygen utilization. Glucose and

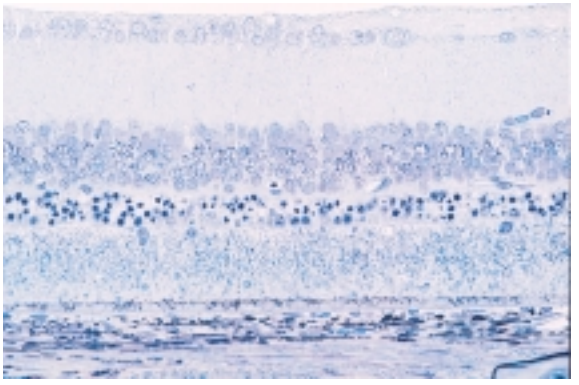

FIGURE 7A
48-day RCS rat. Stained section for comparison to B and C (toluidine blue, x400).

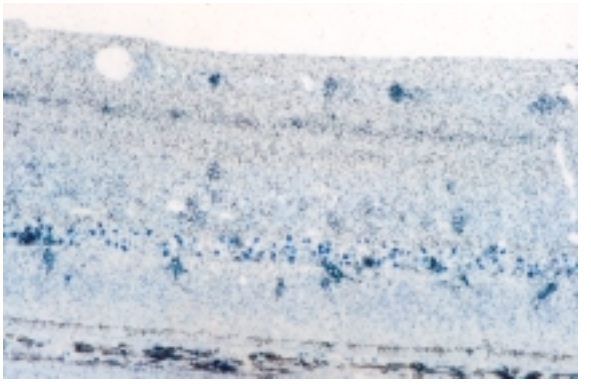

FIGURE 7B
48-day RCS rat. Lightly stained autoradiogram showing change of pattern of 2-DG uptake so that there is much less 2-DG uptake at junction of outer plexiform layer and inner nuclear layer (toluidine blue, x400).

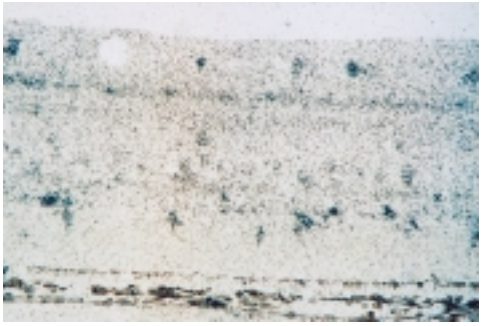

FIGURE 7C
48-day RCS rat. Unstained autoradiogram showing preservation of bands in inner plexiform layer and 2-DG uptake by ganglion cells and cells in photoreceptor layer (unstained, x400).

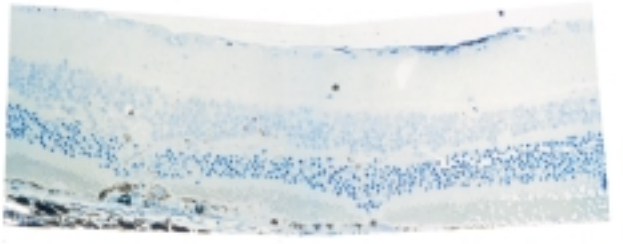

FIGURE 8A
Effect of photocoagulation. Photocoagulation burns are evident as focal areas of loss of photoreceptors and RPE hyperplasia (toluidine blue, x400, montage).

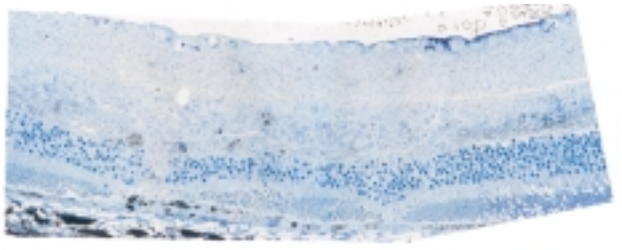

FIGURE 8B
Effect of photocoagulation. Lightly stained autoradiogram showing loss of 2-DG uptake at junction of inner nuclear layer and outer plexiform layer in region of photocoagulation. There is persistent 2-DG uptake at this site at periphery of montage, outside area of photocoagulation (toluidine blue, x400, montage).

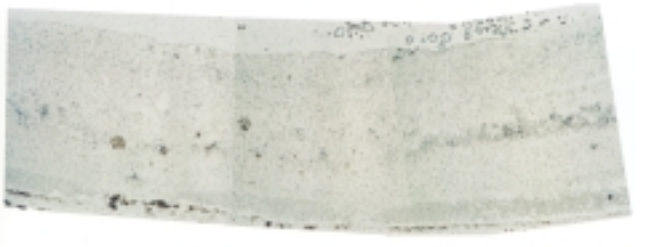

FIGURE 8C
Unstained autoradiogram showing features similar to those shown in 8B (unstained, x400, montage).

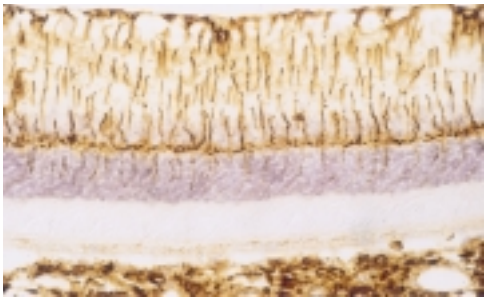

FIGURE 9A
Vimentin staining of Müller cells. Note linear extensions of Müller cells across inner plexiform layer and outer nuclear layer (DAB, x400).

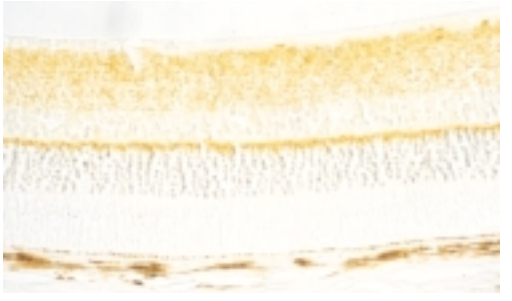

FIGURE 9B
Synaptophysin: There is prominent staining of outer plexiform and inner plexiform layers (DAB, x400).

oxygen are the principal substrates of energy metabolism in the retina, so one would expect some correlative findings. Interestingly, in the perfused rabbit retina, Ames found that inhibition of neurotransmission did not affect the inner retinal oxygen consumption. Similarly, Cringle and coworkers,[38,39] working in vivo in the RCS rat, found that the inner retinal oxygen tension profiles were unaffected by photoreceptor degeneration. Both of these findings are consistent with the hypothesis that neurotransmission though the inner retina is supported by glycolysis, so that loss of neurotransmission would reduce glucose uptake but have no effect on oxygen consumption.

The findings in this study support the concept that there is an interaction between the glucose metabolism of the inner retina and that of the outer retina. This interaction could be important in the changes in the retinal blood vessels following photoreceptor degeneration from retinal dystrophy or from photocoagulation. In the central nervous system, capillary density has been correlated with glucose utilization in some areas of the brain. Furthermore, glial cells (astrocytes and Müller cells) have been shown to direct and guide the vascularization of the inner retina during development.[42,43] It is possible that changes in Müller cell metabolism following loss of the photoreceptors are responsible for the changes in the retinal vessels known to occur with photoreceptor loss.

SUMMARY

This study shows that in vivo glucose uptake by the inner retina is not uniform. The distribution pattern of glucose uptake appears to be consistent with greatest glucose uptake by the Müller cells and at sites of synapses. Following photoreceptor degeneration, there is a marked decrease in the amount of glucose uptake at the junction of the inner plexiform and outer nuclear layers. Decreased glucose uptake in this area is consistent with decreased glucose uptake by Müller cells following photoreceptor atrophy.

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