BRAIN RESEARCH

Research Report

Diabetes changes ionotropic glutamate receptor subunit expression level in the human retina

Ana R. Santiago[a], John M. Hughes[b,c], Willem Kamphuis[d],
Reinier O. Schlingemann[b,c], António F. Ambrósio[a,e,*]

[a]Center for Neuroscience and Cell Biology, Department of Zoology, University of Coimbra, Coimbra, Portugal
[b]Ocular Angiogenesis Group, Department of Ophthalmology, Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands
[c]Ocular Angiogenesis Group, Department of Cell Biology and Histology, Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands
[d]Netherlands Institute for Neuroscience (NIN)-KNAW, an institute of the Royal Netherlands Academy of Arts and Sciences, Department of Cellular Quality Control, Amsterdam, The Netherlands
[e]Center of Ophthalmology of Coimbra, Institute of Biomedical Research on Light and Image (IBILI), Faculty of Medicine, University of Coimbra, Coimbra, Portugal

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ABSTRACT

Early diabetic retinopathy is characterized by changes in subtle visual functions such as contrast sensitivity and dark adaptation. The outcome of several studies suggests that glutamate is involved in retinal neurodegeneration during diabetes. We hypothesized that the protein levels of ionotropic glutamate receptor subunits are altered in the retina during diabetes. Therefore, we investigated whether human diabetic patients have altered immunoreactivity of ionotropic glutamate receptor subunits in the retina. In total, 12 donor eyes from subjects with diabetes mellitus were examined and compared to 6 eyes from non-diabetic subjects without known ocular disease, serving as controls. Immunohistochemical analysis was performed using specific antibodies directed against the ionotropic α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate (AMPA) receptor subunits GluR1, GluR2, GluR4, and against the N-methyl-D-aspartate glutamate receptor subunit NR1. In the inner plexiform and outer plexiform layers the immunoreactivity of GluR2 and NR1 subunits was significantly increased in subjects with diabetes when compared to the levels found in controls. No significant changes in GluR1 and GluR4 subunit expression were observed.

These results suggest that early visual dysfunction in diabetic patients may be due, at least partially, to changes in glutamate receptor subunit expression or distribution.

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1. Introduction

Diabetic retinopathy (DR) is a leading cause of blindness in working-age adults in developed countries. The vascular changes that occur in DR are well documented, and include loss of pericytes and endothelial cells, the formation of microaneurysms, basement membrane thickening and blood-retinal barrier breakdown (Cai and Boulton, 2002). In addition to the vascular

alterations, changes in the neural retina during diabetes have also been reported (Abu El-Asrar et al., 2007; Barber, 2003; Lieth et al., 2000), and these alterations may account for loss in contrast sensitivity and color vision and for alterations in the electro-retinogram (Roy et al., 1986; Sokol et al., 1985).

The initiating mechanisms by which diabetes causes vision loss are still not clearly understood, but the excitatory neurotransmitter glutamate has been implicated. It was shown that both the metabolism and the concentration of glutamate in the retina are altered after short-term experimental diabetes. Diabetic rat retinas are less able to convert glutamate into glutamine (Lieth et al., 1998) and the concentration of glutamate in the retina of streptozotocin (STZ)-induced diabetic rats is increased (Kowluru et al., 2001). Also, glutamate levels are increased in the vitreous of patients with proliferative DR (Ambati et al., 1997), suggesting increased levels in the retina. Moreover, the high-affinity L-glutamate/L-aspartate transporter (GLAST) is impaired in retinal Müller cells isolated from STZ-induced diabetic rats, probably due to oxidation of the glutamate transporter (Li and Puro, 2002).

Glutamate receptors are divided into two major classes of receptors: ionotropic and metabotropic. The ionotropic glutamate receptors are further subdivided into three groups: α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA), kainate (KA) and N-methyl-D-aspartate (NMDA) receptors. While the metabotropic receptors are single polypeptides linked to G-proteins, the ionotropic receptors are multimeric complexes, with AMPA receptors being assembled from subunits designated GluR1–4; kainate receptors from subunits GluR5–7, KA1, and KA2; and NMDA receptors from NR1, NR2A–D, and NR3A subunits. The ion channels associated with NMDA receptors are highly permeable to Ca^{2+}, whereas AMPA and kainate receptor-associated channels are usually less permeable to Ca^{2+}. However, certain combinations of subunits increase the Ca^{2+}-permeability of AMPA and kainate receptor-linked channels. For example, AMPA receptors lacking the GluR2 subunit are Ca^{2+}-permeable (Jensen et al., 1998; Oguro et al., 1999).

Glutamate is the major excitatory neurotransmitter in the central nervous system, including the retina (Ozawa et al., 1998). Photoreceptors, bipolar cells and ganglion cells release glutamate to mediate the transfer of visual signals from the retina to the brain. In the mammalian retina, ionotropic glutamate receptor subunits have been shown to have a widespread and differential distribution throughout the retina (Brandstatter and Hack, 2001; Brandstatter et al., 1998; Thoreson and Witkovsky, 1999; Yang, 2004).

Unregulated glutamate levels are a potential causal factor in a variety of central nervous system disorders (Cull-Candy et al., 2001; Osborne and Herrera, 1994; Sucher et al., 1991). In addition to altered glutamate levels, changes in the distribution and functional characteristics of glutamate receptors may also alter glutamate neurotransmission in the retina during diabetes. In fact, previously, we have showed that ionotropic glutamate receptor subunit expression is changed in cultured retinal neural cells exposed to elevated glucose concentration (Santiago et al., 2006), and Ng et al. reported the upregulation of NR1 and GluR2/3 subunits in ganglion, amacrine and bipolar cells as well as in the inner and outer plexiform layers in the retina of four and 16 weeks diabetic rats (Ng et al., 2004).

In this work, we investigated whether diabetes changes the protein expression levels of ionotropic glutamate receptor subunits in the human retina.

2. Results

The effect of diabetes on the protein expression of ionotropic glutamate receptor subunits was analyzed in human postmortem
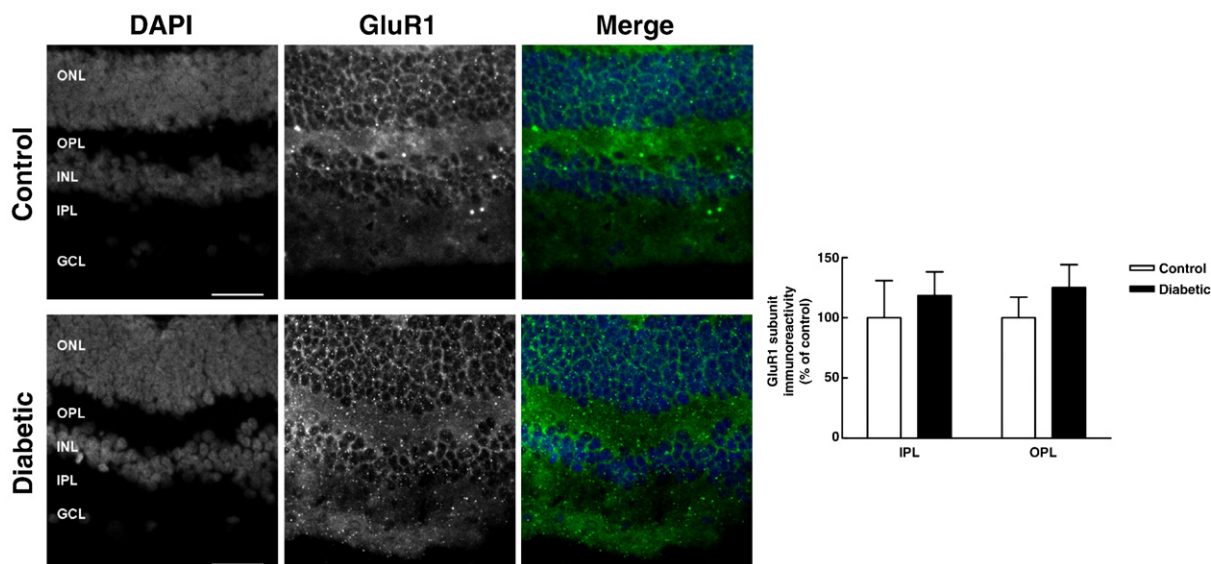

Fig. 1 – GluR1 subunit immunoreactivity in control (upper panel, open bars) and diabetic (lower panel, black bars) human retinas. All pictures were obtained with identical exposure times for control and diabetic retinas, and the pictures shown are representative images. The densitometric measurements were performed using ImageJ software, and the average pixel gray values over the selected area are expressed in percentage of control. Abbreviations: ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Bar: 20 μm.
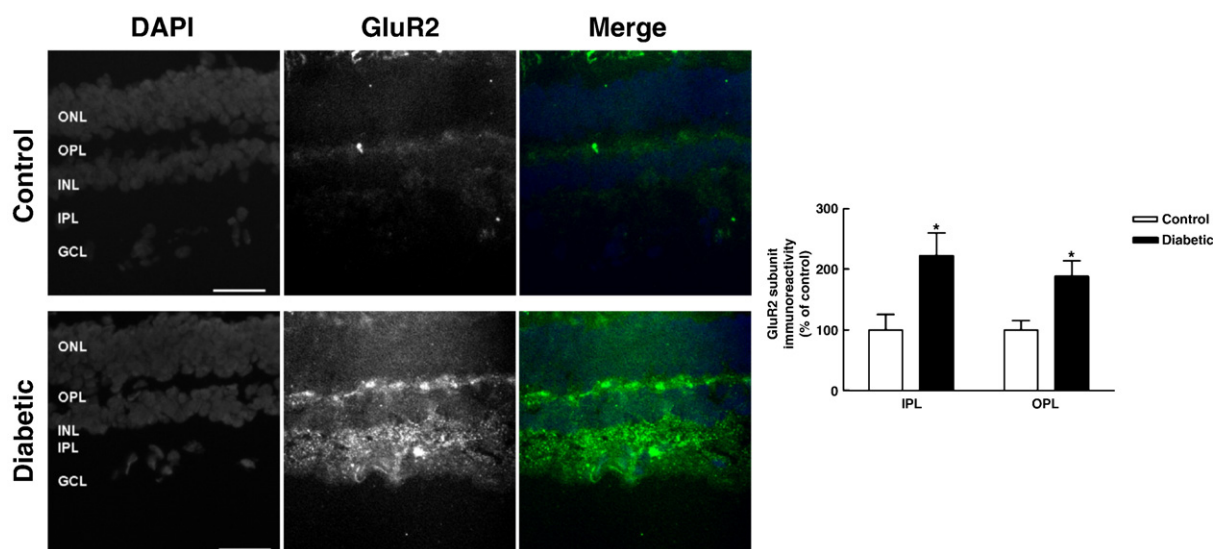
Fig. 2 – GluR2 subunit immunoreactivity in control (upper panel, open bars) and diabetic (lower panel, black bars) human retinas. All pictures were obtained with identical exposure times for control and diabetic retinas, and the pictures shown are representative images. The densitometric measurements were performed using ImageJ software, and the average pixel gray values over the selected area are expressed in percentage of control. Abbreviations: ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. *$p < 0.05$, significantly different from control; two-tailed Student's t-test. Bar: 20 µm.

retinas by immunohistochemistry. There was no difference in age between controls and diabetic patients (72±4 years in controls and 68±3 years in diabetic patients), neither in the postmortem time to enucleation (7.5±0.6 h in controls and 6.5±1.1 h in diabetic patients) (Table 1).

The protein expression pattern of ionotropic glutamate receptor subunits in the retina of mammals has been documented before (Brandstatter, 2002; Brandstatter and Hack, 2001; Brandstatter et al., 1998; Hamassaki-Britto et al., 1993; Muller et al., 1992; Peng et al., 1995). The presentation of results will therefore focus primarily on diabetes-associated alterations. Densitometric analysis for the immunoreactivity of each antibody used was performed in the IPL and OPL.

2.1. GluR1 subunit

The distribution of immunoreactivity of GluR1 subunit in control and diabetic human retinas is shown in Fig. 1. GluR1

Fig. 3 – GluR4 subunit immunoreactivity in control (upper panel, open bars) and diabetic (lower panel, black bars) human retinas. All pictures were obtained with identical exposure times for control and diabetic retinas, and the pictures shown are representative images. The densitometric measurements were performed using ImageJ software, and the average pixel gray values over the selected area are expressed in percentage of control. Abbreviations: ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Bar: 20 µm.
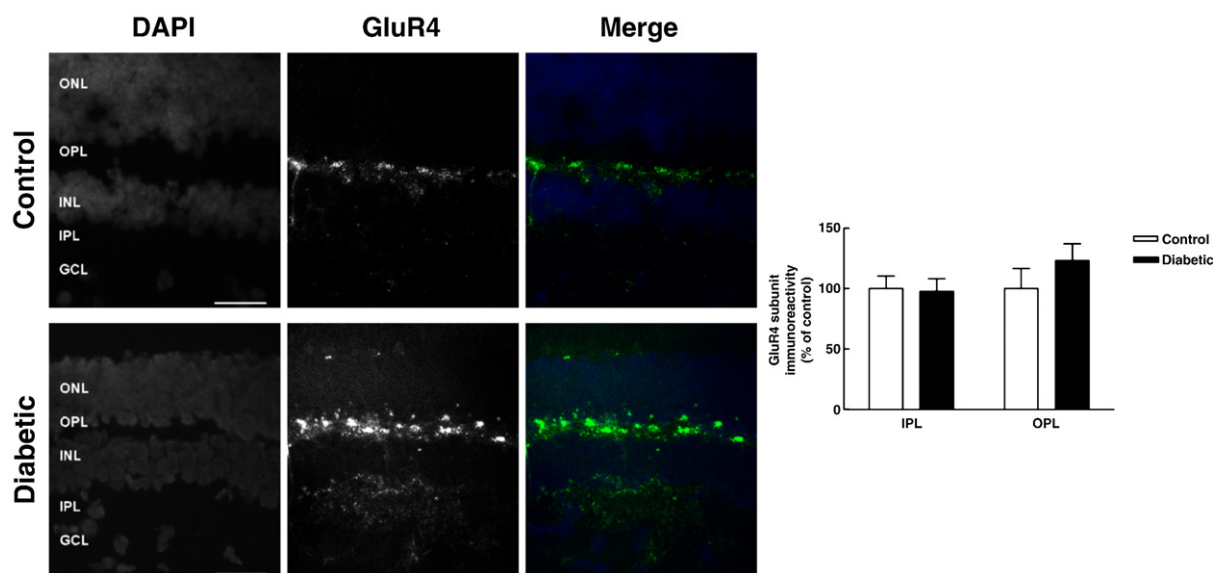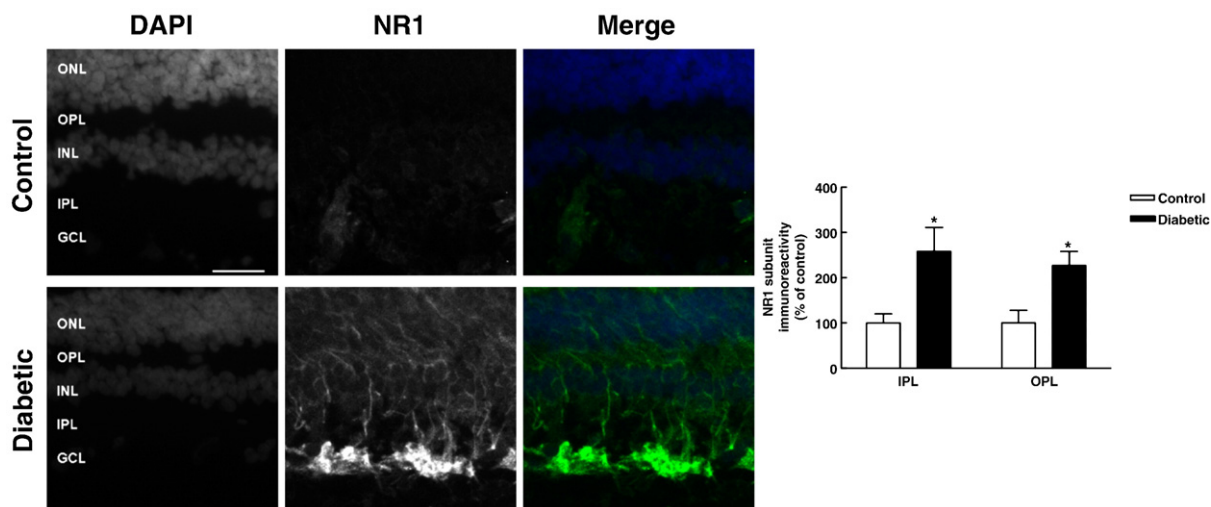
Fig. 4 – NR1 subunit immunoreactivity in control (upper panel, open bars) and diabetic (lower panel, black bars) human retinas. All pictures were obtained with identical exposure times for control and diabetic retinas, and the pictures shown are representative images. The densitometric measurements were performed using ImageJ software, and the average pixel gray values over the selected area are expressed in percentage of control. Abbreviations: ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. *$p < 0.05$, significantly different from control; two-tailed Student's t-test. Bar: 20 μm.

subunit immunoreactivity was found in both plexiform layers and in INL, ONL and GCL. Immunoreactivity in control and diabetic retinas did not differ significantly.

2.2. GluR2 subunit

In control retinas, most of the GluR2 subunit immunoreactivity was observed in OPL (Fig. 2). Weaker immunoreactivity was found in IPL. The GCL was almost devoid of immunoreactivity.

In diabetic retinas, GluR2 subunit immunoreactivity increased in OPL, as compared to the controls, and a strong immunoreactivity was found in IPL with a punctuate appearance, homogenously distributed. Stronger immunoreactivity was also observed in GCL in diabetic human retinas.

Densitometric analysis of the GluR2 subunit immunoreactivity in OPL and IPL confirmed the increase in immunoreactivity in both layers in diabetic retinas. In IPL, immunoreactivity increased to 222±37% ($p < 0.05$) and in the OPL to 188±26% ($p < 0.05$), compared to control group.

2.3. GluR4 subunit

The GluR4 subunit distribution was more restricted than that of the other AMPA glutamate receptor subunits. Immunoreactivity of GluR4 subunit (Fig. 3) was confined to the plexiform layers, more to the OPL. The immunoreactivity patterns observed in the IPL and OPL did not change in diabetic patients compared to controls.

2.4. NR1 subunit

In control retinas, most of the NR1 subunit immunoreactivity was observed in GCL (Fig. 4). A moderate level of immunoreactivity was also observed in IPL.

In diabetic retinas, NR1 subunit immunoreactivity was found in all retinal layers. The intensity of the immunoreactivity was noticeably increased, particularly in the GCL. Densitometric analysis showed a significant increase in NR1 subunit immunoreactivity to 258±53% ($p < 0.05$) and 227±31% ($p < 0.05$) of the control in IPL and OPL, respectively.

3.   Discussion

The main purpose of this study was to investigate whether diabetes changes the expression of ionotropic glutamate receptor subunits in the human retina. Our results show that diabetic human retinas without manifest signs of DR have increased immunostaining of GluR2 and NR1 subunits, indicating elevated tissue levels of these proteins. We did not find detectable changes in the immunoreactivity of GluR1 and GluR4 subunits in human diabetic patients.

AMPA receptors are important mediators of fast excitatory neurotransmission in the retina. AMPA receptors are less permeable to calcium than NMDA receptors. However, AMPA receptors lacking the GluR2 subunit have increased calcium permeability (Jensen et al., 1998; Kondo et al., 2000; Oguro et al., 1999). In general, the expression pattern of the different glutamate receptor subunits is in line with the patterns described in mammals by others (Brandstatter, 2002; Brandstatter and Hack, 2001; Brandstatter et al., 1998; Hamassaki-Britto et al., 1993; Muller et al., 1992; Peng et al., 1995). In contrast to their findings, we observed immunoreactivity for GluR1 subunit in the ONL, suggesting that some axons and cell bodies of photoreceptors contain GluR1 subunits. A similar result, in goldfish retina, was previously reported (Vandenbranden et al., 2000).

The increase in the protein levels of GluR2 subunit in the retina of diabetic rats is in accordance with several other

Table 1 – Subjects characteristics

Case #	Age	Sex	Type of diabetes	Cause of death	Death to enucleation time (h)
Control subjects					
192	75	M		Heart disease	7
203	79	M		Heart disease	10
232	75	M		CVA	8
444	68	M		Heart disease	6
450	79	M		Heart disease	8
740	54	M		Heart disease	6
Diabetic subjects					
277	78	M	DM2 (NID)	CVA	4
288	70	M	DM1	Heart disease	15
321	78	M	DM2 (ID)	Heart disease	11
335	68	F	DM1	Heart disease	7
342	69	M	DM1	CVA	5
346	63	M	DM2 (ID)	Heart disease	2
430	77	F	DM1	Heart disease	1
479	47	M	DM1	Other	9
626	66	F	DM2 (NID)	Heart disease	5
692	66	M	DM1	Heart disease	7
730	77	F	DM2 (ID)	Heart disease	5
738	58	M	DM2 (ID)	Heart disease	7

DM1 = type 1 diabetes; DM2 = type 2 diabetes; NID = non-insulin-dependent; ID = insulin-dependent; CVA = cerebral vascular accident; Heart disease = coronary artery disease, heart failure or heart arrhythmias.

observations. Using an in vitro model of DR, we have shown that GluR2 subunit expression is also increased in high glucose-treated rat retinal cells and that calcium homeostasis is deregulated (Santiago et al., 2006). Ng et al. (2004) have shown an increase in GluR2/3 subunit expression in ganglion, amacrine and bipolar cells as well as in the inner and outer plexiform layers in one and four weeks STZ-induced diabetic rats. In addition, the *ob/ob* mouse, a model of type 2 diabetes, was found to have increased binding sites for AMPA and NMDA receptors in the gray matter of the spinal cord (Li et al., 1999). In the brain of STZ-diabetic rats the binding properties of AMPA receptors and the expression of NMDA receptor subunits were also altered (Di Luca et al., 1999; Gagne et al., 1997). The functional implications of the higher content of the GluR2 subunit in diabetic subjects are not yet clear but it may be speculated that it will decrease the Ca^{2+}-permeability of AMPA receptor-associated channels.

Our observations on increased NR1 subunit immunoreactivity in the diabetic human retina are in accordance with previous findings in diabetic rats (Ng et al., 2004). The NMDA receptor channels provide an important route for Ca^{2+} entry, and subsequent activation of Ca^{2+}-dependent intracellular enzymes. Neurotoxicity associated with excitatory amino acids was reported to be mediated to a large extent through the activation of NMDA receptors (Romano et al., 1995; Vorwerk et al., 1996). In the context of diabetes, the increase of NR1 subunit expression and of retinal glutamate content may therefore contribute to the retinal neuronal loss observed in diabetes (Aizu et al., 2002; Barber et al., 2005, 1998; Martin et al., 2004; Park et al., 2003; Santiago et al., 2007). However, within the complex regulation of glutamate signaling and metabolism in the retina, several mechanisms may counteract the damaging effect brought upon by

increased NR1 levels. Desensitization of the glutamate-mediated response is an important physiological property of the AMPA receptors, and the upregulation of GluR2 may be a compensatory response to limit the possible damage caused by excessive glutamate levels (Jayakar and Dikshit, 2004). Previously, it was already reported that the increase of NR1 subunit protein level is accompanied by an increase in the levels of the calcium-binding proteins, calbindin and parvalbumin (Ng et al., 2004). These proteins function as buffer in order to maintain the calcium homeostasis and to protect cells against the potentially damaging effects of excessive calcium influx during overexcitatory activity.

In conclusion, the observed changes in ionotropic glutamate receptor subunit expression show that diabetes changes a main component of excitatory neurotransmission, suggesting a potential impairment of synaptic communication throughout the retina, which may contribute to eventual color vision defects or loss of contrast sensitivity.

4. Experimental procedures

4.1. Retinal tissue

Donor eyes were obtained and used in the study in accordance with the provisions of the Declaration of Helsinki for research involving human tissue. Human donor eyes, rejected for corneal transplantation because of corneal opacities or abnormalities of corneal endothelium, were obtained from the Cornea Bank, Amsterdam. No donor details were revealed, other than sex, age, postmortem time and cause of death. In total, 12 donor eyes from subjects with diabetes mellitus but without manifest diabetic retinopathy, according to ophthalmological examination, and 6 eyes from non-diabetic subjects without known ocular disease serving as control subjects were examined. Details about the donors are given in Table 1.

4.2. Immunohistochemistry

Intact eyes were snap frozen in liquid nitrogen cooled in isopentane and stored at −80 °C until used. Tissue blocks of the posterior half of the frozen human globes were cut starting at the base of the optic nerve, extending anteriorly to the mid-periphery of the eye using a sterile razor blade. Serial cryostat sections (10 μm thick) of both control and diabetic patients were placed on the same slide in order to minimize methodological variation during immunostaining. Sections were air dried and fixed in 2% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.0) for 10 min at room temperature and then rinsed with PB for 30 min. Sections were then incubated for 1 h in 0.05 M PB (pH 7.0)

Table 2 – Primary antibodies used in this study

Primary antibody	Dilution	Source [a]
Rabbit anti-GluR1	1:100	Chemicon International
Goat anti-GluR2	1:750	Santa Cruz Biotechnology, Inc.
Rabbit anti-GluR4	1:750	Chemicon International
Rabbit anti-NR1	1:100	Chemicon International

[a] Location: Chemicon International, Temecula, CA, USA; Santa Cruz Biotechnology, Santa Cruz, CA, USA.

containing 10% normal goat serum (NGS), 0.4% Triton X-100, and 1% bovine serum albumin, at room temperature. Sections were subsequently incubated overnight with primary antibody (listed in Table 2) diluted in 0.05 M PB containing 3% NGS, 0.4% Triton X-100, and 1% BSA, at room temperature. Negative controls were included, by omitting the primary antibody, but these never yielded immunoreactivity patterns.

Sections were incubated with the secondary antibodies for 1 h in 1% NGS at room temperature. Immunoreactivity was visualized using indocarbocyanine (Cy3)-conjugated affinity-purified F(ab′)2 secondary antibodies, either goat anti-rabbit, or rabbit anti-goat IgG (1:600; Jackson Immuno Research; Brunschwig Chemie, Amsterdam, The Netherlands). Sections were washed and coverslipped in Vectashield mounting medium with DAPI (Vector Laboratories, Amsterdam, The Netherlands). The preparations were visualized with a microscope (Axioshop 2 Plus; Carl Zeiss Meditec, Inc.), coupled to a camera (Axiocam HRc; Carl Zeiss Meditec., Inc.). Microscope settings and exposure times were kept identical for all the control and diabetic retinas to obtain an accurate representation of the differences in immunoreactivity intensities. Adobe Photoshop was used to scale and arrange the TIFF files for presentation. Densitometric analysis was performed using the public domain ImageJ program (http://rsb.info.nih.gov/ij/) in the plexiform layers, OPL and IPL, because these are the regions where the glutamatergic synapses are located, and, in addition, these layers can be more easily defined for quantification purposes.

4.3. Statistical analysis

From each subject, 3 sections were stained and at least 3 images were randomly acquired from each section. For each image, the background mean gray value was obtained from the biggest region possible without cells and was subtracted from the mean gray value obtained for each retinal layer analyzed.

Results are presented as mean±SEM. Statistical analysis was performed using the two-tailed Student's t-test.

Acknowledgments

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