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Diabetes causes transient changes in the composition and phosphorylation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and interaction with auxiliary proteins in the rat retina

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Purpose: The impairment of glutamatergic neurotransmission has been associated with diabetic complications in the central nervous system, such as diabetic retinopathy. Here, we investigated the effect of elevated glucose exposure and diabetes on α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor composition, subunit phosphorylation, and the association of the GluA2 subunit with accessory proteins in the retina.
Methods: The subunit composition of AMPA receptors and the association of the GluA2 subunit with modulatory proteins were evaluated with coimmunoprecipitation in retinal neural cell cultures and in the retina of experimentally induced-diabetic rats. The phosphorylation status of AMPA receptor subunits was evaluated with western blotting.
Results: In retinal neural cell cultures, elevated glucose did not significantly alter the composition of AMPA receptors, namely, the interactions between the GluA1, GluA2, and GluA4 subunits, but reduced GluA2 association with GRIP1. Moreover, elevated glucose did not cause changes on the level of GluA1 phosphorylated at serine residues 831 and 845. Diabetes induced early transitory changes in the interaction between AMPA receptor subunits GluA1, GluA2, and GluA4. At 8 weeks of diabetes, the content of GluA1 phosphorylated at serine 831 or serine 845 in the retina increased, compared to age-matched controls.
Conclusions: Taken together, these results suggest that diabetes induces dynamic changes in AMPA receptor subunit composition, which could affect glutamatergic transmission in the rat retina.

Diabetic retinopathy is the most common complication of diabetes mellitus, the leading cause of legal blindness in working-age adults [1,2]. Although traditionally considered a microvascular disease, diabetic retinopathy also affects retinal neurons [3,4]. In fact, loss in contrast sensitivity, color vision abnormalities, and changes in oscillatory potentials in electroretinograms have been observed in diabetic rats and patients and constitute early signs of neural dysfunction [5-8]. Moreover, neural cells of the retina undergo apoptosis in human diabetic patients and in animal models of diabetes [9,10]. Diabetes-induced apoptosis in neurons occurs mainly in the inner layers of the retina, suggesting amacrine and ganglion cell degeneration, and is accompanied by a decrease in the retinal thickness [11-13]. In fact, retinal neurodegeneration occurs early in diabetic retinopathy, and targeting neuroprotection as a therapeutic approach would be an important strategy [reviewed in 14]. Hyperglycemia is considered the main trigger for the development of diabetic complications, namely, diabetic retinopathy, and a good glycemic control has been shown to decrease the rate of progression of these diseases [15-17]. The main excitatory neurotransmitter in the retina is glutamate. α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors are ionotropic glutamate receptors that mediate fast excitatory neurotransmission in the central nervous system. These receptors consist of four subunits (GluA1–4) that combine to form tetramers [18]. The functional characteristics of AMPA receptors are defined by their subunit composition and interaction with auxiliary proteins [19-22]. In particular, the Q/R edited form of the GluA2 subunit determines important functional properties of AMPA receptor channels, such as rectification behavior and calcium permeability [20,23]. This editing of GluA2 also affects the assembly and trafficking of the receptors to the synapse [24]. Trafficking and localization of GluA2-containing receptors involve interaction with associated proteins, which is regulated by phosphorylation events on this subunit [25,26]. The C-terminal domain of the GluA2

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subunit is the interaction site with several cytosolic proteins, such as GRIP/ABP, PICK1, NSF, and AP2. GRIP1 and PICK1 can also interact with the GluA3 and GluA4 (short tail) subunits. The GluA1 subunit interacts with different proteins, namely, 4.1 and SAP97 [reviewed in 27]. The C-terminal domain of the subunits is also the site for phosphorylation by several kinases such as protein kinase C (PKC), protein kinase A (PKA), and calcium calmodulin kinase II [28-36].

The effect of diabetes on the levels and physiology of glutamate receptors in the central nervous system is far from being clarified. Some reports have shown that diabetes increases the mRNA expression of several subunits of ionotropic glutamate receptors in the central nervous system. The GluN1 and GluN2B subunits of NMDA receptors are increased in the hippocampus, cortex, cerebellum, and dorsal horn of diabetic rats [37-40]. The gene expression of the GluA1, GluA2, and GluA3 subunits of AMPA receptors is also increased in the dorsal horn of diabetic rats [39]. In the retina, NMDA and AMPA receptor subunits are upregulated in diabetic rats [41,42]. In particular, the expression of the GluA2 subunit of AMPA receptors is upregulated in the inner retinal layers of diabetic rats and patients [41,43,44]. In addition, in cultured retinal neurons, exposure to an elevated concentration of glucose has been shown to increase GluA2 levels [45,46]. In this study, we sought to evaluate whether diabetes and hyperglycemic conditions alter the composition, phosphorylation status, and interaction with regulatory proteins of AMPA receptors in the rat retina.

METHODS

Primary culture of rat retinal neural cells: Rat pups were handled according to the European Union (EU) guidelines for the use of experimental animals (86/609/EEC). The experiments were approved by our Institutional Ethics Committee (Ethics Commitee of the Faculty of Medicine of the University of Coimbra). Retinal neural cell cultures were obtained from 3- to 5-day-old Wistar rats. The retinas were dissected in Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution (in mM: 137 NaCl, 5.4 KCl, 0.45 KH_2PO_4, 0.34 Na_2HPO_4, 4 $NaHCO_3$, and 5 glucose, pH 7.4) under a dissection microscope. The retinas were digested with 0.1% trypsin (GIBCO Invitrogen, Life Technologies, Paisley, Scotland) for 15 min at 37 °C. After digestion, the cells were collected by centrifugation at 190 ×*g*, and the pellet was resuspended in Eagle´s minimum essential medium (Sigma-Aldrich, St. Louis, MO) supplemented with 26 mM $NaHCO_3$, 25 mM HEPES, 10% heat-inactivated fetal bovine serum (GIBCO Invitrogen), penicillin (100 U/ml), and streptomycin (100 g/ml). Cells were plated at a density of 2×10^6 cells/cm^2 on poly-D-lysine (0.1 mg/ml) coated culture dishes. These retinal neural cell cultures are mixed cultures composed mainly of neurons, particularly amacrine cells, but also contain some photoreceptors, rod bipolar cells, and horizontal cells, as well as glial cells [47-49].

The cells were maintained at 37 °C in a humidified incubator with 5% CO_2 and 95% air for 7 days and incubated with 30 mM glucose for 24 h and 48 h. For osmotic control, cells were incubated with 25 mM D-mannitol (plus 5 mM glucose from the culture medium) for the same time periods.

Animals: The animals were treated in agreement with the EU guidelines for the use of experimental animals (86/609/EEC). Diabetes was induced with a single intraperitoneal injection of 65 mg/kg streptozotocin (STZ; freshly dissolved in citrate buffer; pH 4.5) in 8-week-old male Wistar rats and was confirmed 2 days later by blood glucose levels exceeding 250 mg/dl with a glucometer (Ascencia Elite, Bayer Portugal, Carnaxide, Portugal). Diabetic and age-matched control animals were euthanized under anesthesia (2.5% isoflurane inhalation in 1 l/min O_2 using a gas-anesthetizing system [VetEquip, Pleasanton, CA]) follwed by cervical dislocation at 2,4 or 8 weeks after the onset of diabetes.

Preparation of total cellular extracts:

Retinal neural cell culture extracts—Cells were washed twice in cold PBS (in mM: 137 NaCl, 2.7 KCl, 1.8 KH_2PO_4 and 10 Na_2HPO_4, pH 7.4) and lysed with radioimmunoprecipitation assay (RIPA; in mM: 150 NaCl, 50 Tris, 5 EGTA, 1% Triton X-100, 0.5% DOC, 0.1% sodium dodecyl sulfate [SDS]) supplemented with complete-mini protease inhibitor cocktail tablets (Roche, Basel, Switzerland), 1 mM DTT, 50 mM NaF, and 1 mM Na_3VO_4. The cells were scrapped, and the lysates were stored at −80 °C. After thawing, the samples were centrifuged at 16,000 ×*g* for 10 min, the supernatants were collected, and the protein concentration was determined with the bicinchoninic acid (BCA) colorimetric assay.

Whole retina extracts—The retinas were rapidly dissected and rinsed in cold PBS. Then the tissue was homogenized in RIPA, sonicated six times (for 1 s each), and centrifuged at 16,000 ×*g* for 10 min. Protein concentration in the supernatants was determined with the BCA colorimetric assay.

All samples were denaturated with 6× concentrated denaturating buffer (0.5 M Tris-HCl pH 6.8, 30% glycerol, 10% SDS, 0.6 M dithiothreitol, 0.02% bromophenol blue) at 95 °C for 5 min and stored at −20 °C until use.

Co-immunoprecipitation assay: Cells were scrapped and lysed in immunoprecipitation buffer (in mM: 10 Tris, pH

7.0, 100 NaCl, 2 EDTA, 2 EGTA, 1% Triton X-100) supplemented with complete-mini protease inhibitor cocktail tablets, 50 mM NaF, and 1 mM Na_3VO_4, and centrifuged at 16,000 ×*g* for 10 min at 4 °C. The retinas were homogenized in immunoprecipitation buffer, sonicated, and centrifuged at 16,000 ×*g* for 10 min at 4 °C.

The protein concentration of the lysates was determined with the BCA colorimetric assay. Protein concentration was equalized in all samples, and 500 µg of total protein was incubated with the antibodies (see Table 1) overnight at 4 °C under mild shaking. Protein A sepharose (100 µl, GE Healthcare, Buckinghamshire, UK; 1:1 slurry in immunoprecipitation buffer) was added to each sample and incubated for 2 h at 4 °C under mild shaking. The protein complexes were precipitated with centrifugation at 16,000 ×*g* for 1 min. Pellets were washed three times with washing buffer (in mM: 10 Tris, pH 7.0, 100 NaCl, 2 EDTA, 2 EGTA, 1% Triton X-100), and the final pellet was resuspended in 2× denaturating buffer boiled at 95 °C for 5 min.

Western blot analysis: To detect phosphorylated proteins, 100 µg of total protein was loaded, and to detect immunoprecipitated proteins, the samples were loaded in equal volumes. Samples were separated in 4–8% SDS-polyacrylamide gel (SDS–PAGE) and then transferred electrophorectically to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA). The membranes were blocked for 1 h at room temperature in Tris buffered saline (TBS, in mM: 137 NaCl, 20 Tris-HCl; pH 7.6) containing 0.1% Tween-20 (TBS-T) and 5% low-fat milk or 5% BSA to detect phosphorylated proteins. The membranes were incubated with the primary antibody (listed in Table 1) for 2 h at room temperature. After washing for 1 h in TBS-T, the membranes were incubated for 1 h at room temperature with the respective secondary antibody conjugated with alkaline phosphatase (GE Healthcare), prepared in TBS-T with 1% low-fat milk or 5% BSA. The immunoreactive bands were visualized with the Enhanced Chemi-Fluorescence system (ECF, GE Healthcare) and imaging system (Storm 860, Molecular Dynamics, GE Healthcare). Digital quantification of band intensity was performed using ImageQuant 5.0 software (Molecular Dynamics, GE Healthcare).

Statistical analysis: The results are presented as mean ± standard error of the mean (SEM). The data were analyzed using the Student *t* test or one-way ANOVA, followed by Dunnett's post-hoc test. The differences between the means were considered significant for values of $p<0.05$. The statistical analysis was performed in Prism 5.0 software (GraphPad Software, San Diego, CA).

RESULTS

Effect of elevated glucose on the composition and phosphorylation of AMPA receptors and on interaction with modulatory proteins in retinal neural cells: Hyperglycemia is considered the main trigger of diabetic complications. We have previously shown that elevated glucose induces an increase in the total protein levels of GluA2 in retinal neural cells in culture [45,46]. According to the several time points studied previously, the major increase in GluA2 protein content was detected after 2 days of exposure to elevated glucose. Therefore, we chose this time point to investigate whether exposure to elevated glucose alters the composition of AMPA receptors in retinal neural cell cultures. For this, we exposed the retinal cells to 30 mM glucose or mannitol (osmotic control) for 48 h, and with coimmunoprecipitation studied the protein association between different AMPA receptor subunits. The lack of a suitable anti-GluA3 antibody prevented us from studying

Table 1. List of primary antibodies.

Primary antibody	Retinal neural cells		Retina		Source
	WB	IP	WB	IP	
Rabbit anti-GluA1	2.125	2.5 µg	2.125	2.5 µg	Millipore
Rabbit anti-GluA2	1:3000	2.5 µg	1:500	2.5 µg	Millipore
Rabbit anti-GluA4	1:500	2.5 µg	1:1000	2.5 µg	Millipore
Rabbit anti-GRIP1	1:1000	3 µg	1:500	3 µg	Millipore
Rabbit anti-NSF	1:500	2.5 µg	1:500	2.5 µg	Abcam
Rabbit anti-pS831GluA1	1:500	———	1:500	———	Millipore
Rabbit anti-pS845GluA1	1:500	———	1:500	———	Abcam
Rabbit antipS880GluA2	———	———	1:500	———	Abcam
Mouse anti-β-actin	1:20000	———	———	———	Sigma

WB: western blot; IP: immunoprecipitation

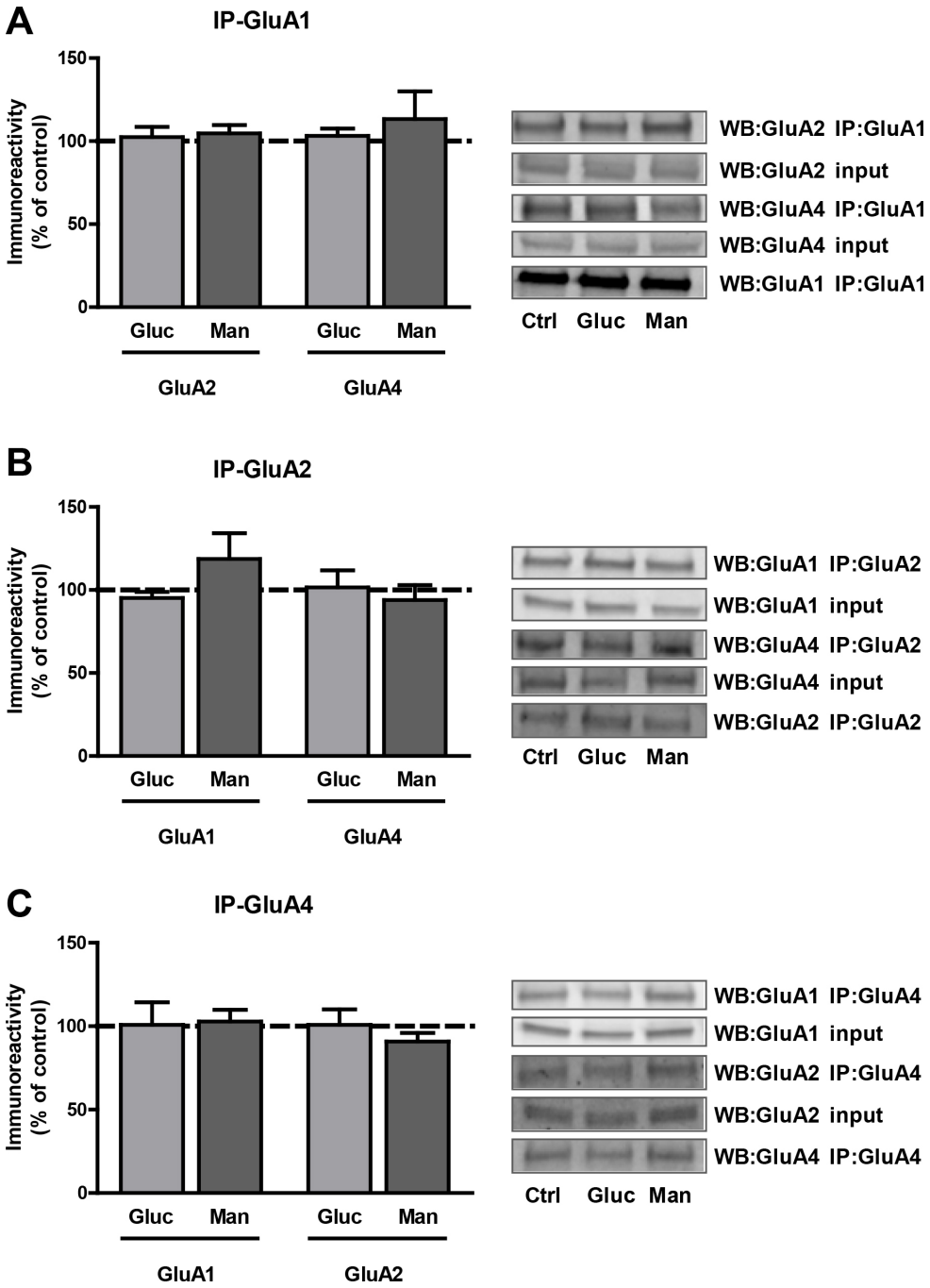

Figure 1. Elevated glucose does not alter the composition of AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors in cultured retinal neural cells. Cells were exposed to 30 mM glucose (Gluc) or 25 mM mannitol (Man - osmotic control; + 5 mM glucose) for 48 h. The interaction between AMPA receptor subunits was analyzed with coimmunoprecipitation (IP) followed by western blotting (WB). **A**: Immunoreactivity of GluA2 and GluA4 in the immunoprecipitated samples of GluA1. **B**: Immunoreactivity of GluA1 and GluA4 in the immunoprecipitated samples of GluA2. **C**: Immunoreactivity of GluA1 and GluA2 in the immunoprecipitated samples of GluA4. The results are presented as mean ± standard error of the mean (SEM) of at least three independent experiments and are expressed as a percentage of the control. Representative immunoblots are presented on the right of the respective graphs.

the protein interactions with this subunit. We did not detect changes in the association levels between the GluA1, GluA2, and GluA4 subunits in retinal neural cells exposed to elevated glucose (Figure 1). Mannitol also did not change the association levels between the subunits studied (Figure 1).

AMPA receptor subunits are direct substrates of kinases, and their phosphorylation at specific residues regulates the channel properties of the receptor. Moreover, the phosphorylation of AMPA receptor subunits can regulate interaction with associated proteins that modulate the membrane trafficking and synaptic targeting of the receptors [27]. Therefore, we investigated the effect of elevated glucose on the phosphorylation of GluA1 subunit at two serine residues, serine 831 and serine 845, and the phosphorylation of GluA2 subunit at serine 880, in retinal neural cell cultures. Elevated glucose exposure for 24 h and 48 h did not alter the phosphorylation status of GluA1 in retinal neural cells at either serine 831 or serine 845 (Figure 2). We did not detect immunoreactivity for pS880-GluA2 with western blot in extracts from the control cells or the elevated glucose-treated cells.

We also investigated the effect of elevated glucose on the interaction of GluA2 with the GluA2-containing receptors associated proteins, GRIP1 and NSF. Elevated glucose significantly decreased GRIP1 bound to GluA2 in retinal neural cell cultures ($p<0.05$, one-way ANOVA followed by Dunnett's post-hoc test, Figure 3A). However, when we immunoprecipitated GRIP1, the decrease in the levels of GluA2 associated with GRIP1 did not reach significance in the high glucose-treated cells (Figure 3B). This discrepancy in the results related to the immunoprecipitation of GluA2 or GRIP1 could be due to differences in the efficacy of the antibodies used in the experiment. The decrease in the interaction of GluA2 and GRIP1 was not present in the mannitol-treated cells indicating that it was not induced by the increase in osmolarity (Figure 3A,B). The association levels of GluA2 with NSF were not affected by exposure to elevated glucose in retinal cells (Figure 3A,C).

Effect of diabetes on the composition, phosphorylation, and interaction with modulatory proteins of AMPA receptors in the rat retina: We also investigated the effect of diabetes on AMPA receptor composition, phosphorylation, and interaction with associated proteins in the retina. Since we were interested in the early stages of the disease, we investigated AMPA receptor composition at 2, 4, and 8 weeks of diabetes duration in the STZ-induced diabetes model.

When GluA1-containing receptors were immunoprecipitated with a specific antibody against GluA1, we detected a decrease in the interaction with GluA4 at 8 weeks of diabetes ($p<0.001$, Student t test, Figure 4A). Additionally, we detected an increase in the interaction of the GluA2 subunit with the GluA1 and GluA4 subunits at 2 and 4 weeks of diabetes for GluA1 and at 2 and 8 weeks of diabetes for GluA4, respectively ($p<0.05$, Student *t* test, Figure 4B). When the GluA4-containing receptors were immunoprecipitated, we did not

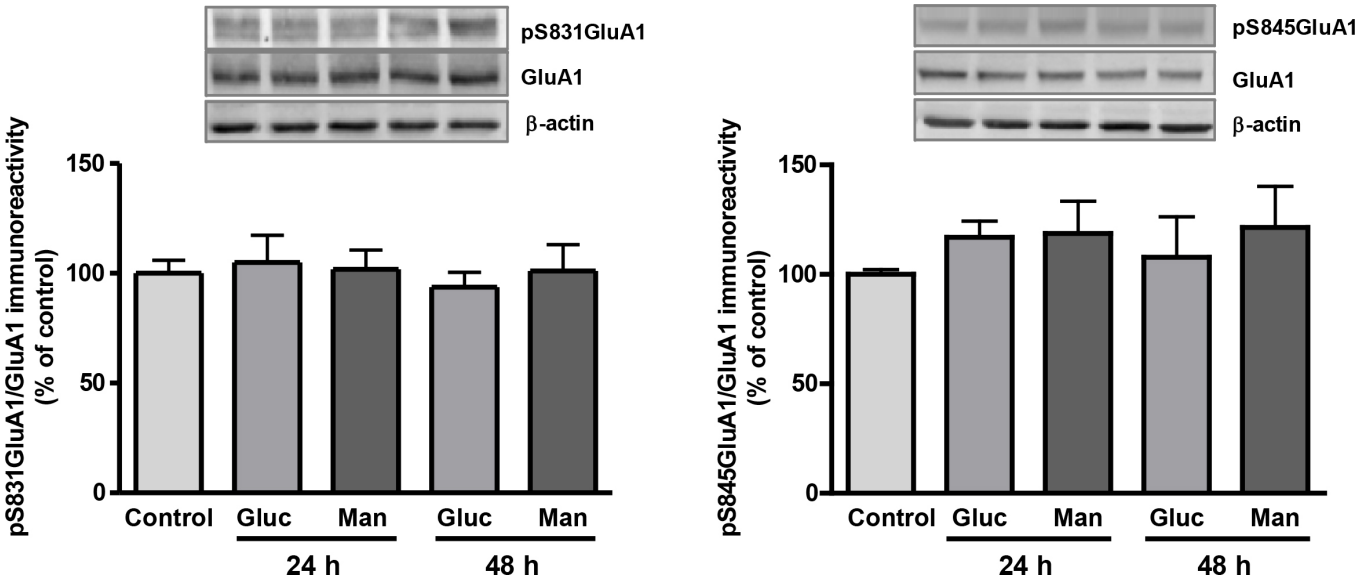

Figure 2. Elevated glucose does not change GluA1 phosphorylation at serine 831 (pS831GluA1) and 845 (pS845GluA1) in retinal neural cells. Cells were exposed to 30 mM glucose (Gluc) or 25 mM mannitol (Man - osmotic control; + 5 mM glucose) for 24 h or 48 h. Immunoreactivity was analyzed with western blotting. The results are presented as the mean ± standard error of the mean (SEM) of at least four independent experiments and are expressed as a percentage of the control. Representative immunoblots are presented above the graphs.

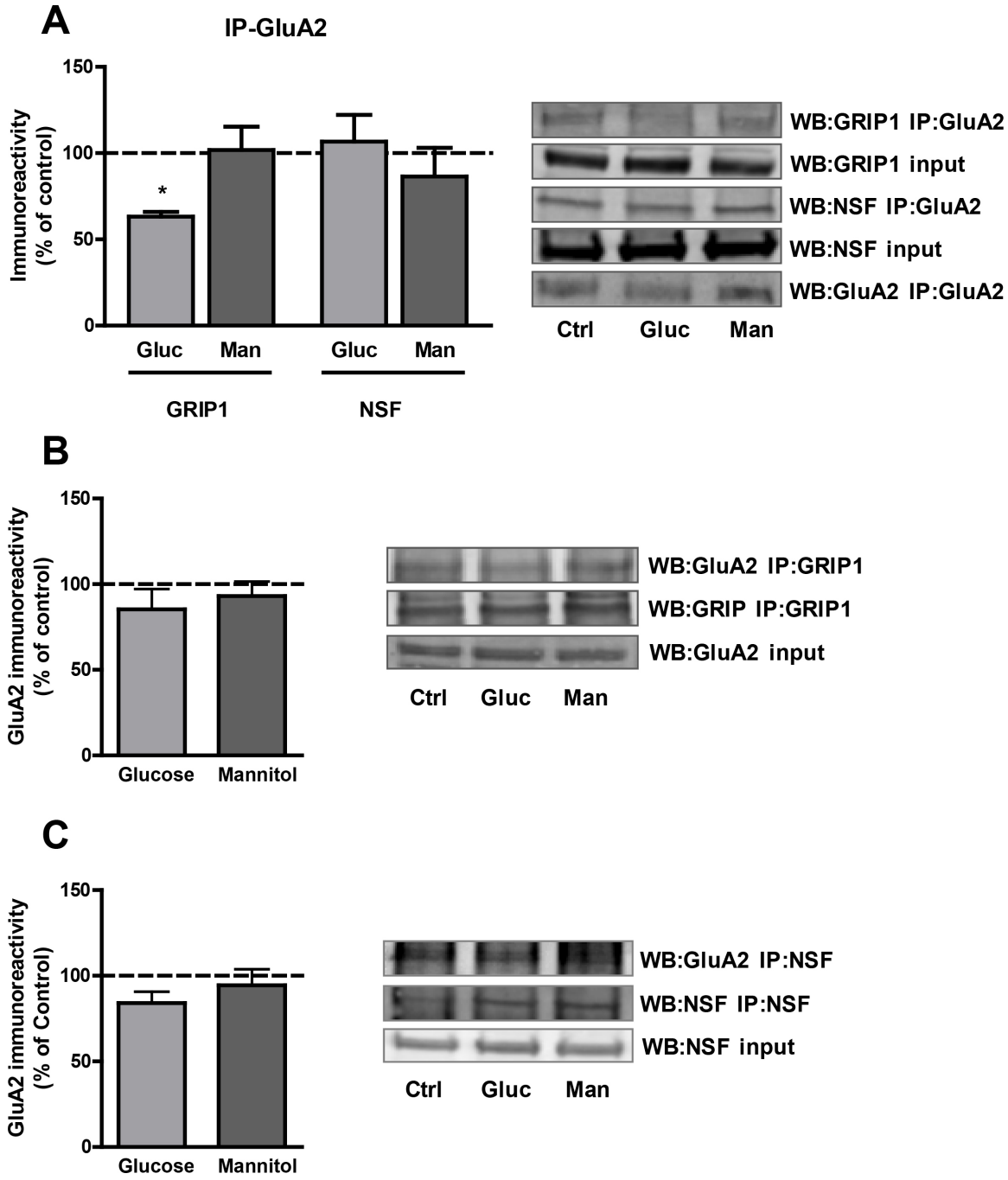

Figure 3. Elevated glucose decreases the interaction between GluA2 subunit and GRIP1 in retinal neural cells. Cells were exposed to 30 mM glucose (Gluc) or 25 mM mannitol (Man - osmotic control; + 5 mM glucose) for 48 h. The interaction between the GluA2 subunit and its interacting proteins was analyzed with coimmunoprecipitation (IP) followed by western blotting (WB). **A**: Immunoreactivity of GRIP1 and NSF in the immunoprecipitated samples of GluA2. **B**: Immunoreactivity of GluA2 in the immunoprecipitated samples of GRIP1. **C**: Immunoreactivity of GluA2 in the immunoprecipitated samples of NSF. The results are presented as the mean ± standard error of the mean (SEM) of at least three independent experiments and are expressed as a percentage of the control. Representative immunoblots are presented at the right of the respective graphs. *p<0.05, significantly different from the control as determined with one-way ANOVA followed by Dunnett's post hoc test.

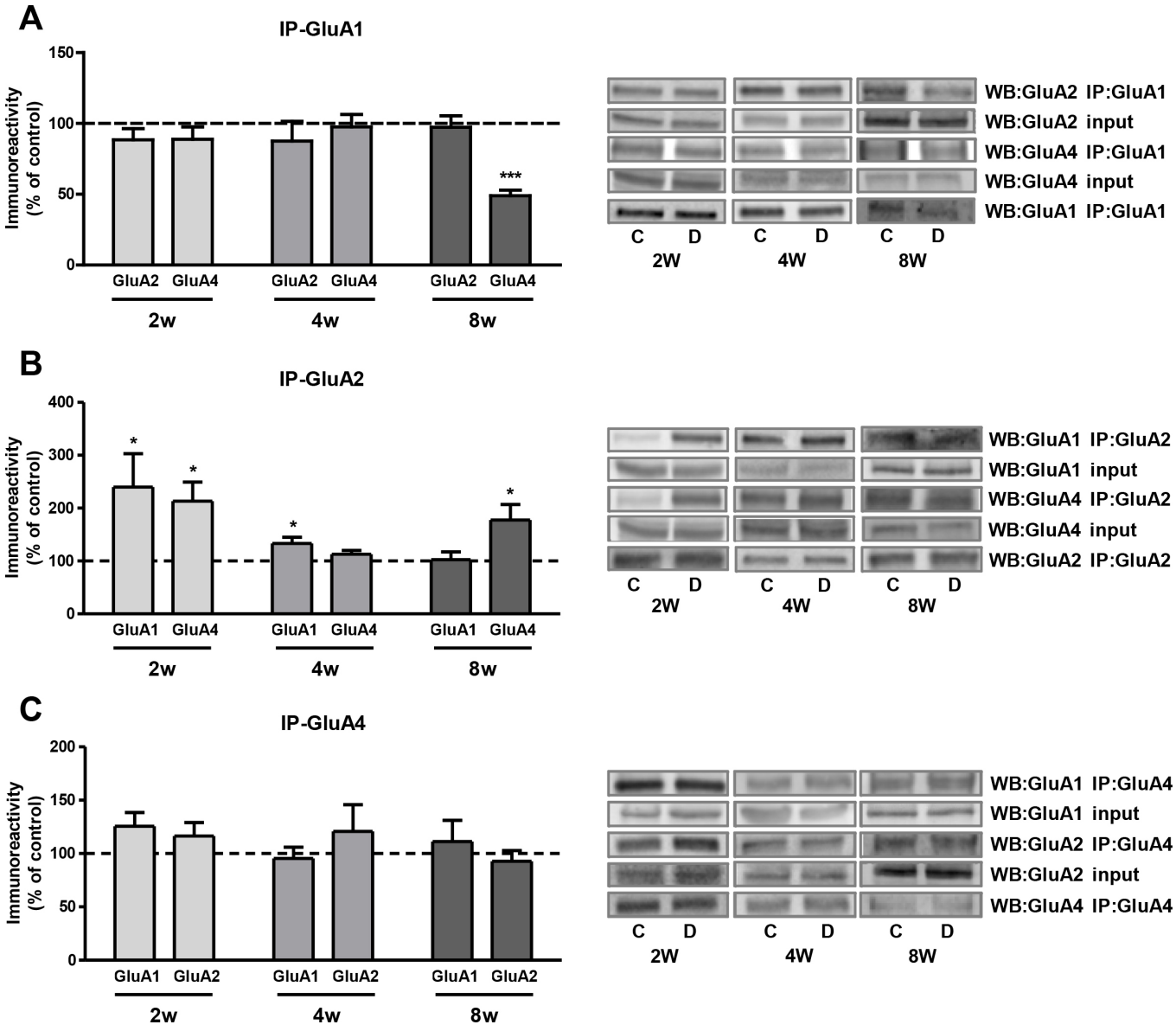

Figure 4. Effect of diabetes on the composition of AMPA receptors in the retina. Diabetes (2, 4, or 8 weeks' duration) was induced by a single intraperitoneal injection of streptozotocin in Wistar rats. Th interaction between AMPA receptor subunits was analyzed with coimmunoprecipitation (IP) followed by western blotting (WB). **A**: Immunoreactivity of GluA2 and GluA4 in the immunoprecipitated samples of GluA1. **B**: Immunoreactivity of GluA1 and GluA4 in the immunoprecipitated samples of GluA2. **C**: Immunoreactivity of GluA1 and GluA2 in the immunoprecipitated samples of GluA4. The results are presented as the mean ± standard error of the mean (SEM) of at least three independent experiments (at least six retinas from three animals) and are expressed as a percentage of the control. Representative immunoblots are presented at the right of the respective graphs. *p<0.05, ***p<0.001, significantly different from the control as determined with the Student t test.

detect significant changes in the association levels with the other subunits (Figure 4C). As for experiments with cultured cells, in the retinas some results related to the interaction between the subunits did not match when one or the opposite subunit was immunoprecipitated with the specific antibodies for the two subunits. As previously described, this discrepancy could be due to different efficacies of the antibodies for immunoprecipitation.

As in retinal cell cultures, we also studied the phosphorylation status of GluA1 and GluA2 in the retinas of diabetic rats. The ratio of GluA1 subunits phosphorylated at serine 831 or serine 845 to total GluA1 increased at 8 weeks of diabetes

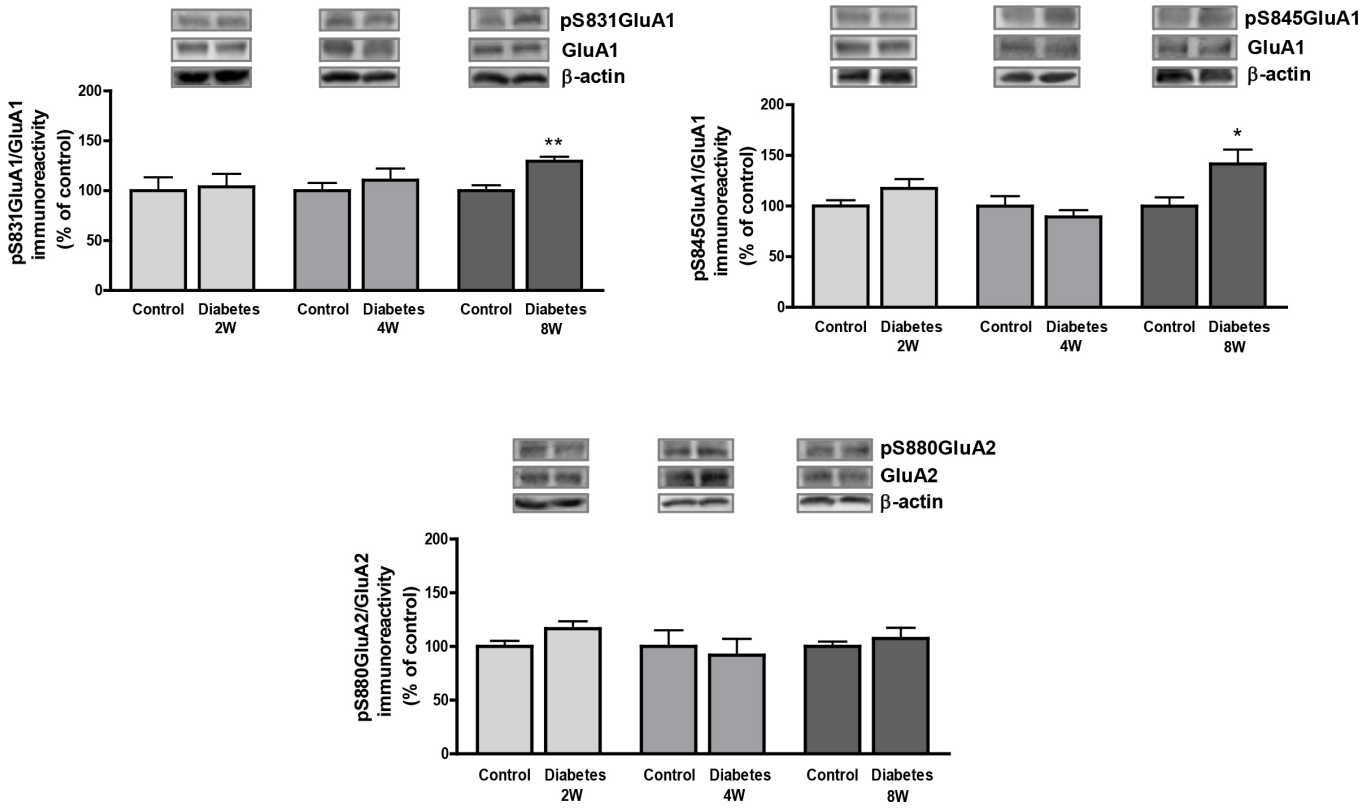

Figure 5. Diabetes increases the phosphorylation of GluA1 subunit in the retina. Diabetes (2, 4, or 8 weeks' duration) was induced by a single intraperitoneal injection of streptozotocin in Wistar rats. Immunoreactivity was analyzed with western blotting. The results are presented as the mean ± standard error of the mean (SEM) of at least three independent experiments (at least six retinas from three animals) and are expressed as a percentage of the control. Representative immunoblots are presented above the graphs. *p<0.05; **p<0.01, significantly different from control as determined with the Student *t* test.

compared to the age-matched controls (p<0.01 and p<0.05, Student t test, Figure 5). No changes were detected in the ratio of GluA2 phosphorylated at serine 880 to total GluA2 (Figure 5). Moreover, no significant changes were detected in the interactions of GluA2 with the associated proteins GRIP1 and NSF at any time point of diabetes studied (Figure 6).

DISCUSSION

Diabetes alters the glutamate levels in the retina of diabetic rats and in the vitreous of patients with proliferative diabetic retinopathy [50-52]. The uptake of glutamate in the retina is also impaired by diabetes [53,54]. In addition, we and others have shown in different models, such as experimentally induced diabetes, patients, and cell cultures exposed to elevated glucose, that the content of several subunits of ionotropic glutamate receptors in the retina is altered [41-46]. Together, this evidence indicates that the glutamatergic system is impaired by diabetes in the retina, which may contribute to the changes observed in electroretinograms and contrast sensitivity and color perception in humans.

However, despite this evidence, it is still unknown whether AMPA receptor composition, subunit phosphorylation, and interaction with modulatory proteins are affected by diabetes or hyperglycemic conditions. To our knowledge, only one study has investigated the effect of diabetes on AMPA receptor trafficking. Semkova and colleagues showed that experimentally induced-diabetes increases the levels of GluA2 phosphorylated at serine 880 and does not alter the total content of PICK1 in the retina, suggesting increased internalization of GluA2-containing receptors [43]. Even in other disease models, little is known concerning the effects on AMPAR composition and trafficking. In primary neuronal cell cultures, a decrease in the surface expression of AMPA receptors and a decrease in the basal levels of pS845GluA1 by polyQ-huntingtin and by amyloid-beta oligomers were recently demonstrated [55,56].

In this study, in retinal neural cell cultures, we did not detect significant changes in the composition of the AMPA receptors, namely, in the association between the subunits GluA1, GluA2, and GluA4, suggesting that elevated glucose

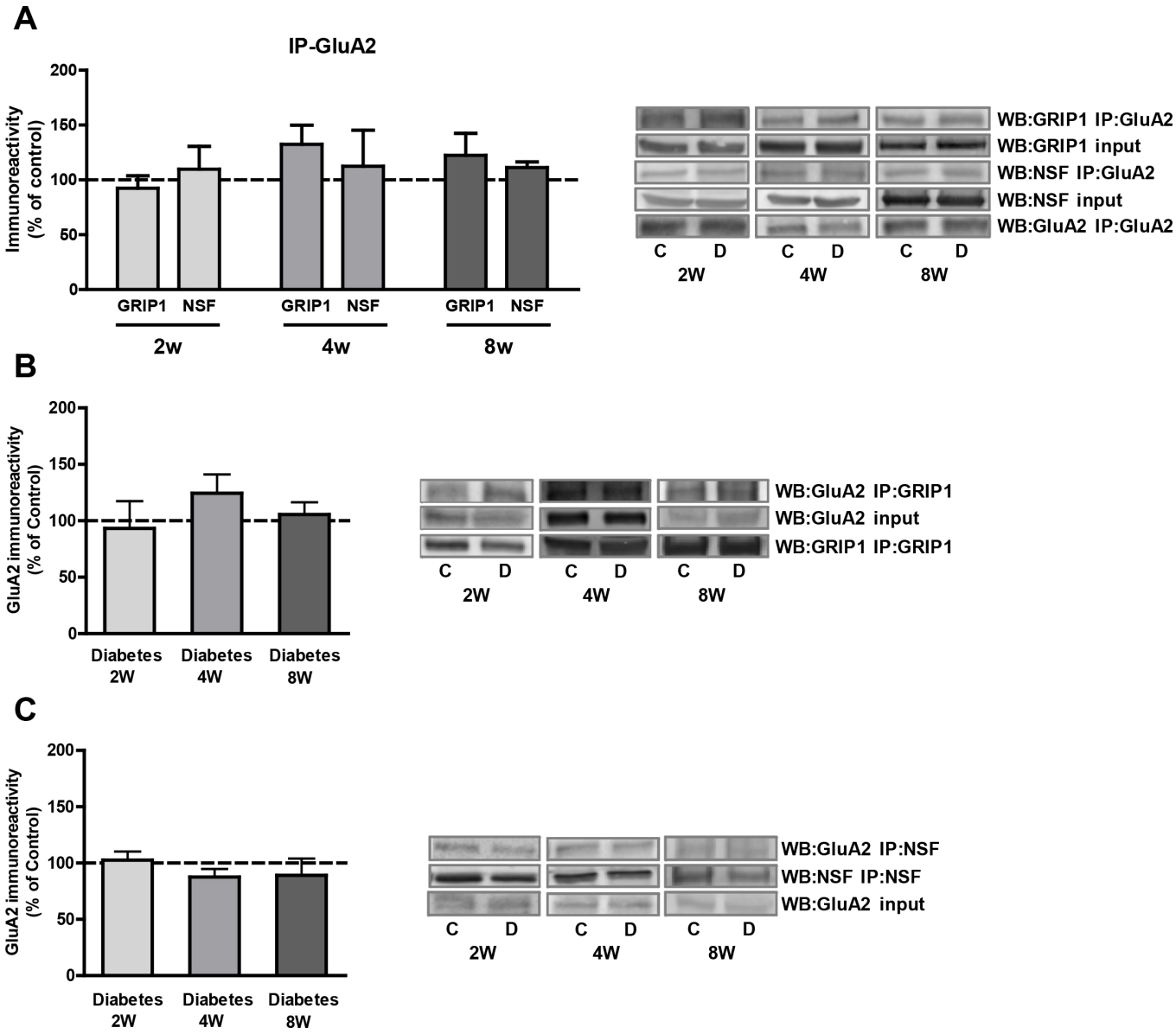

Figure 6. Diabetes does not change the interaction of GluA2 subunit with GRIP1 or NSF in the retina. Diabetes (2, 4, or 8 weeks' duration) was induced by a single intraperitoneal injection of streptozotocin in Wistar rats. The protein interaction between GluA2 subunit and its interacting proteins was analyzed with coimmunoprecipitation followed by western blotting. **A**: Immunoreactivity of GRIP1 and NSF in the immunoprecipitated samples of GluA2. **B**: Immunoreactivity of GluA2 in the immunoprecipitated samples of GRIP1. **C**: Immunoreactivity of GluA2 in the immunoprecipitated samples of NSF. The results are presented as the mean ± standard error of the mean (SEM) of at least three independent experiments (at least six retinas from three animals) and are expressed as a percentage of the control. Representative immunoblots are presented at the right of the respective graphs.

does not induce changes in the composition of AMPA receptors in cultured cells, at least for the time points studied in this work. In the same cultures, we found an increase in the total GluA2 subunit levels in cells exposed to elevated glucose, without changes in the total GluA1 or GluA4 content for up to 4 days of exposure [46]. This could indicate an increase in GluA2/GluA3 heteromeric receptors. The lack of a suitable antibody against GluA3 prevented us from investigating this hypothesis.

The precise role of PDZ proteins in the recycling of AMPA receptors is still not fully understood. In GluA2-containing AMPA receptors, changes in the GRIP1/ABP and PICK1 interactions with GluA2 after phosphorylation at S880 by PKC seem to be critical for regulated trafficking

of the receptors [57,58]. The model proposed suggests that PKC binding to PICK1 targets the PKC-PICK1 complex to GRIP1/ABP-GluA2 complex. The phosphorylation of GluA2 at serine 880 by PKC prevents the rebound of GRIP1/ABP to GluA2 but not the binding of PICK1 to GluA2 [58]. Therefore, the decreased interaction of GluA2 with GRIP1 induced by elevated glucose in retinal neural cells suggests a decrease in synaptic anchorage of GluA2-containing receptors. Unfortunately, it was not possible to study the interaction of GluA2 to PICK1, since we could not separate PICK1 from the immunoglobulin heavy chain. GRIP1/ABP can also be detected in intracellular compartments, and the differential palmitoylation of GRIP1/ABP determines the intracellular and the plasma membrane-associated portions in hippocampal neurons [59]. In this work, we detected the total interaction GRIP1-GluA2, but we could not distinguish if the observed reduction is at the intracellular or membrane pool. The PICK1-GluA2 complex is also regulated by NSF, which has ATPase activity and disrupts PICK1-GluA2 interactions [60]. NSF association with GluA2 has been shown to be needed to stabilize GluA2-containing AMPA receptors at the plasma membrane, preventing regulated endocytosis of the receptors [33,34,36,61]. Since the association of GluA2 with NSF was not changed by elevated glucose, this observation suggests that stabilization of GluA2-containing receptors at the plasma membrane is preserved, at least at this time point. In fact, the GluA2-containing AMPA receptors at the plasma membrane are increased in retinal neural cells exposed to elevated glucose [46]. Thus, we speculate that elevated glucose does not change the stabilization or anchorage of GluA2-containing AMPA receptors at the plasma membrane but decreases the interaction with GRIP1 in intracellular compartments.

Another important feature that affects AMPA receptor characteristics and recycling is the phosphorylation of the subunits. Phosphorylation of GluA1 at serine 831 by CaMKII leads to an increase in channel conductance of AMPA receptors [62,63]. Phosphorylation of GluA1 at serine 845 has been associated with the stabilization of GluA1-containing receptors at the plasma membrane and increased incorporation of the receptors into the synapse [64-69]. In retinal neural cells, exposure to elevated glucose for 24 or 48 h did not affect the phosphorylation of the GluA1 subunit at serine 831 or serine 845, suggesting that the stabilization of GluA1-containing receptors at the plasma membrane is not affected by exposure to elevated glucose in these cells at the time points studied.

We also investigated the effect of diabetes on AMPA receptor subunit interaction. We found a few changes in the retinas of diabetic rats (some occurred as early as 2 weeks after diabetes onset); however, they were not persistent. These dynamic changes in the protein levels could reflect adaptive changes in the retina along time against the stress induced by diabetes, and thus, the glutamatergic system, similar to many other proteins expressed in the retina, such as exocytotic proteins, responds differently over time to diabetes [42,70,71]. Regarding GluA2 interactions with associated proteins GRIP1 or NSF, no significant changes were detected, suggesting that diabetes does not induce changes in GluA2-containing AMPA receptor trafficking and synaptic targeting in the retina. In fact, PICK1 was described as present throughout the retinal layers and unchanged by 2 and 6 weeks of diabetes in the rat retina [43]. However, since the retinal neural cell cultures are enriched in amacrine cells, and we detected a decrease in the interaction of GluA2 with GRIP1, we cannot exclude the hypothesis that there are cell-specific changes in the trafficking of AMPA receptors that are diluted in the total retina.

The phosphorylation of the GluA1 subunit at serine 831 and 845 was increased in the retina at 8 weeks after the onset of diabetes. This increase suggests that GluA1-containing AMPA receptors are stabilized at the plasma membrane and that the conductance of the channels is increased. Regarding the phosphorylation of GluA2 at serine 880, we did not detect changes in the total retinal extracts at any time point studied, suggesting that no significant changes are expected in the internalization of GluA2-containing AMPA receptors.

Although there is some discrepancy between the results obtained with the two models used in this study (retinal cell cultures and type 1 diabetes rat model), the differences could be related to changes in specific cell types or circuits existing in the retina that are not present in the cell culture model or be related to the contribution from other factors triggered by diabetes in the animal model, which are not present in the cell culture model. For instance, in the cell culture model, Müller cells and certain neuronal cell types are less abundant compared to the retina. Another factor that could contribute to these differences is the lack of the trophic factor insulin in the animal model. In addition, the time of exposure to elevated glucose concentrations is different in both models: relatively short-term exposure in cell cultures compared to the longer periods of hyperglycemia in the animal model.

Regarding the animal model used, streptozotocin has been shown to be neurotoxic upon intracerebroventricular administration [72,73]. However, we did not expect a direct effect of STZ on the effects detected since STZ was administered intraperitoneally and this drug does not cross the blood–brain barrier and is rapidly excreted in urine [74-77]. Overall, our results indicate that elevated glucose and diabetes cause

dynamic changes in AMPA receptor composition and stabilization at the plasma membrane that could affect the synaptic transmission in the retina, which is likely to occur in specific retinal circuits.

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