Universidade de Aveiro Departamento de Biologia
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Efeito da glucose elevada no conteúdo de receptores AMPA na retina e hipocampo

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Effect of high glucose on AMPA receptors content in the retina and hippocampus

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica do Doutor Francisco Ambrósio, Investigador Auxiliar da Faculdade de Medicina da Universidade de Coimbra e co-orientação científica da Doutora Paula Gonçalves, Professora Associada do Departamento de Biologia da Universidade de Aveiro.

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palavras-chave Receptores AMPA, diabetes, glucose elevada, retina, hipocampo.

Resumo

A retinopatia diabética é uma das principais complicações da diabetes *mellitus*, sendo considerada a maior causa de perda de visão nos países ocidentais. A diabetes tem sido também associada a défices moderados em processos cognitivos e de memória, os quais estão associados a alterações no hipocampo.

A hiperglicémia é considerada o principal factor para o desenvolvimento destas complicações e várias evidências sugerem que o conteúdo de receptores ionotrópicos de glutamato pode ser afectado em condições de hiperglicémia. Além disso, resultados obtidos no nosso laboratório em células de retina expostas a glucose elevada, sugerem que há um aumento da subunidade GluR2 nos receptores do ácido alfa-amino-3-hidroxi-5-metil-4-isoxazol–propiónico (AMPA), um subtipo de receptores ionotrópicos de glutamato responsáveis pela neurotransmissão excitatória rápida, que estão localizados na membrana plasmática. Assim, o objectivo deste estudo consistiu em investigar o efeito da glucose no conteúdo de diferentes subunidades dos receptores AMPA na membrana plasmática das células de retina, e também de hipocampo.

Para tal, as culturas primárias de retina e de hipocampo foram expostas a uma concentração elevada de glucose (30 mM), de forma a mimetizar condições de hiperglicémia, e manitol (24,5 mM mais 5,5 mM de glucose), o controlo osmótico, durante dois e sete dias, respectivamente. Para identificar as proteínas presentes na membrana plasmática, foi realizado um ensaio de biotinilação e, de seguida, a expressão proteica das subunidades dos receptores AMPA (GluR1, GluR2 e GluR4) foi determinada por *western blotting*. A immunoreactividade das subunidades dos receptores AMPA foi também avaliada por imunocitoquímica em culturas de células de retina.

Neste estudo verificou-se que a concentração elevada de glucose aumentou os níveis totais e membranares das subunidades GluR1 e GluR2, enquanto os níveis proteicos da subunidade GluR4 aumentaram apenas na membrana plasmática. Os resultados obtidos para as quantidades totais das três subunidades foram confirmados por imunocitoquímica. Contrariamente, no hipocampo a glucose elevada não alterou significativamente os níveis totais e membranares das subunidades dos receptores AMPA. O manitol, controlo osmótico, não alterou a expressão das subunidades dos receptores AMPA.

Estes resultados indicam que a glucose elevada pode alterar os níveis totais e membranares das subunidades dos receptores AMPA em células neuronais de retina, e que este efeito não é devido a um aumento da osmolaridade. As alterações na expressão e distribuição dos receptores AMPA podem ter um impacto na fisiologia da retina. Os neurónios de hipocampo parecem ser mais resistentes a alterações nas subunidades dos receptores AMPA quando expostos a condições de hiperglicémia, o que pode de alguma forma explicar o surgimento de alterações na retina causadas pela diabetes antes dos défices de memória.

Keywords AMPA receptors, diabetes, high glucose, retina, hippocampus.

Abstract

Diabetic retinopathy is a common complication of diabetes *mellitus* and a leading cause of vision loss in western countries. Diabetes has been also associated with moderate impairment in cognitive and memory processes, which have been linked to changes in the hippocampus.

Hyperglycemia is considered the main factor for the development of these complications, and several evidences indicate that the content of ionotropic glutamate receptors can be affected by hyperglycemic conditions. Also, previous results obtained in our laboratory, in retinal cells exposed to high glucose, suggest that α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors (AMPAR), a subtype of ionotropic glutamate receptor responsible for fast excitatory neurotransmission, that are located at the plasma membrane are enriched in GluR2 subunits. Therefore, the main aim of this study was to investigate whether high glucose could affect the content of different AMPAR subunits at the plasma membrane in retinal cells, and also in hippocampal cells.

For this purpose, retinal and hippocampal cell cultures were exposed to high glucose (30 mM), to mimic hyperglycemia, and mannitol (24.5 mM plus 5.5 mM of glucose), the osmotic control, for two and seven days, respectively. To identify the proteins present at the plasma membrane, we performed a biotinylation assay, and then AMPAR subunits (GluR1, GluR2 and GluR4) protein expression was assessed by western blotting. The immunoreactivity of AMPAR subunits was also evaluated by immunocytochemistry in retinal cell cultures.

We demonstrated that high glucose increased both total and surface content of GluR1 and GluR2 subunits in retinal cells, whereas GluR4 protein levels increased only at the plasma membrane. The results obtained for the total amount of AMPAR subunits were confirmed by immunocytochemistry. Contrarily, in hippocampal cultures, high glucose did not change significantly the total and surface levels of AMPAR subunits. Mannitol, the osmotic control, had no effect on the protein levels of AMPAR subunits.

These results indicate that high glucose can alter the total and membrane protein levels of AMPAR subunits in retinal neural cells, and these effects are independent of the increase in osmolarity. Changes in AMPAR expression and distribution can eventually have an impact on retinal physiology. Hippocampal neurons appear to be more resistant than retinal neurons to changes in AMPAR subunits when exposed to hyperglycemic conditions, which may explain the occurrence of retinal dysfunction in diabetes before memory impairments.

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Abbreviations

AMPA – α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid

AMPAR – AMPA receptors

BCA – Bicinchoninic acid

BSA – Bovine serum albumin

CaMKII – Calcium/calmodulin-dependent protein kinase II

CNQX – 6-cyano-7-nitroquinoxaline-2,3-dione

CNS – Central Nervous System

DAPI – 4',6-diamidino-2-phenylindole

DIV – Days in vitro

EATTs – Excitatory amino acid transporters

ECF – Enhanced chemifluorescence

ER – Endoplasmic reticulum

ERG – Electroretinogram

GFAP - Glial fibrillary acidic protein

GRIP – Glutamate receptor-interacting protein

GRIP/ABP – Glutamate receptor-interacting protein/AMPA binding protein

HBSS – Hank's balanced salt solution

iGluRs - Ionotropic glutamate receptors

LTD – Long term depression

LTP – Long term potentiation

MEM – Eagle's minimum essential medium

mGluRs – Metabotropic glutamate receptors

mRNA – Messenger ribonucleic acid

NBQX – 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione

NMDA – N-methyl-D-aspartate

NS3763 – 5-carboxyl-2,4-di-benzamidobenzoic acid

NSF – N-ethylmaleimide-sensitive factor

NTD – N-terminal domain

PBS – Phosphate-buffered saline

PICK1 – Protein interacting with C kinase 1

PKA – Cyclic-AMP-dependent protein kinase

PKC – Protein kinase C

PSD-95 – Postsynaptic density-95 protein

PVDF – Polyvinylidene fluoride

RIL – Reversion induced LIM protein

RNA – Ribonucleic acid

RPE – Retinal pigment epithelium

SDS – Sodium dodecyl sulfate

SEM – Standard error of the mean

STZ – Streptozotocin

TARPs – Transmembrane AMPAR regulatory proteins

TBS-T – Tris-buffer saline-Tween

TUNEL – Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling

UBP296 – (R,S)-3-(2-carboxybenzyl) willardiine

VGLUT – Vesicular glutamate transporter

I – Introduction

1. Glutamate

Glutamate is the major excitatory neurotransmitter in the central nervous system (CNS). An elevated percentage of excitatory neurons in the CNS are glutamatergic, and glutamate is released approximately in half of all brain synapses. Glutamate is a nonessential amino acid that does not cross the blood-brain barrier and for that reason must be synthesized in neurons from local precursors.

The most common precursor of glutamate is glutamine, which is released by glial cells. Glutamine is taken up by neurons via excitatory amino acid transporters (EATTs), where it is deaminated and turned back into glutamate. Glutamate is then loaded into synaptic vesicles via vesicular glutamate transporters (VGLUT). Once released from the presynaptic terminal, glutamate diffuses across the synaptic cleft and then binds to its receptors on the dendrites of the post-synaptic neurons. The action of glutamate in the synaptic cleft is terminated by uptake into neurons and neighboring glial cells via specific transporters (Fig. 1) (Purves, 2004).

Glutamate has also been shown to be involved in certain pathological conditions. High concentrations of glutamate accumulated in the synaptic cleft, as for example after an ischemic episode, promote an excessive activation of glutamate receptors that can lead to the damage of many cell components, and consequently cell death. This phenomenon is usually referred to as glutamate excitotoxicity (Purves, 2004).

1.1. Glutamate receptors

Glutamate is the agonist of two distinct categories of glutamate receptors: metabotropic glutamate receptors (mGluRs), that are coupled to G protein and generate intracellular second messengers (Fig. 2A), and ionotropic glutamate receptors (iGluRs), which have receptor-associated cation channels, that open upon receptor activation by agonists (Fig. 2B).

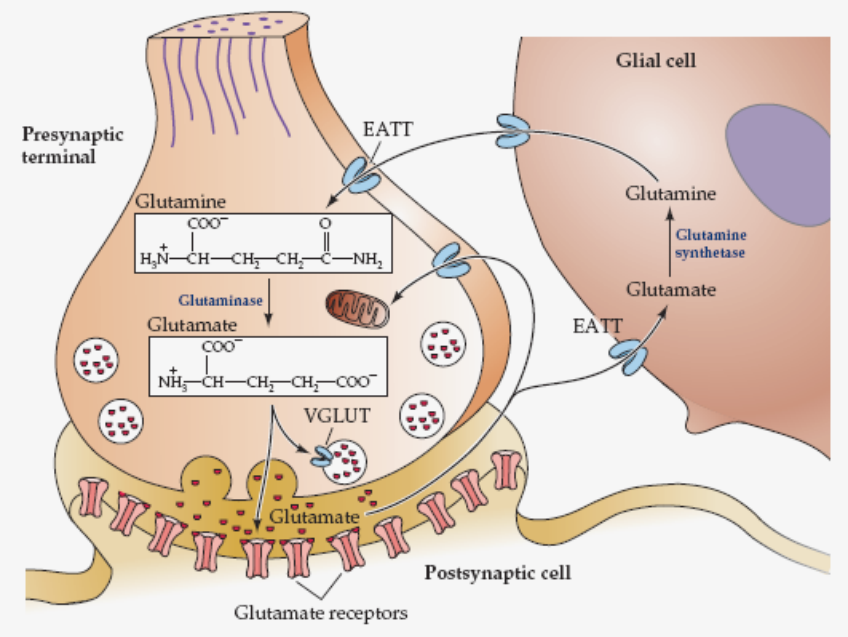

Figure 1 - Schematic representation of glutamate synthesis and cycling between neurons and glia. Glutamate released from the nerve terminal by exocytosis is taken up by excitatory amino acid transporters (EATTs) present at glial cells and neurons. In glial cells, glutamine synthetase converts glutamate into glutamine. Glutamine is subsequently released from the glial cells and taken up by neuronal EATTs. Neurons convert glutamine back to glutamate, which is loaded into synaptic vesicles by VGLUT (adapted from Purves, 2004).

1.1.1. Metabotropic glutamate receptors

Metabotropic glutamate receptors comprise three functional groups, based on amino acid sequence homology, agonist pharmacology and the signal transduction pathways to which they are coupled (Fig. 3A). These receptors are characterized by having seven transmembrane domains and a large N-terminal extracellular domain, which possesses the glutamate binding domain and confers selectivity for agonists. In addition, the second intracellular loop and the intracellular C-terminal domain determine the specificity of G protein coupling (Fig. 2A). Unlike the ionotropic glutamate receptors, mGluRs cause slower postsynaptic responses that can either increase or decrease the excitability of postsynaptic cells.

1.1.2. Ionotropic glutamate receptors

Ionotropic glutamate receptors, a family of ligand-gated ion channels, are responsible for the majority of fast excitatory neurotransmission in the CNS. There are three types of ionotropic glutamate receptors, classified based on their pharmacological, molecular and electrophysiological properties: Kainate, *N*-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors (Fig. 3B). Each type contains several homologous subunits that assemble in a specific manner into tetrameric complexes (Fig. 2B).

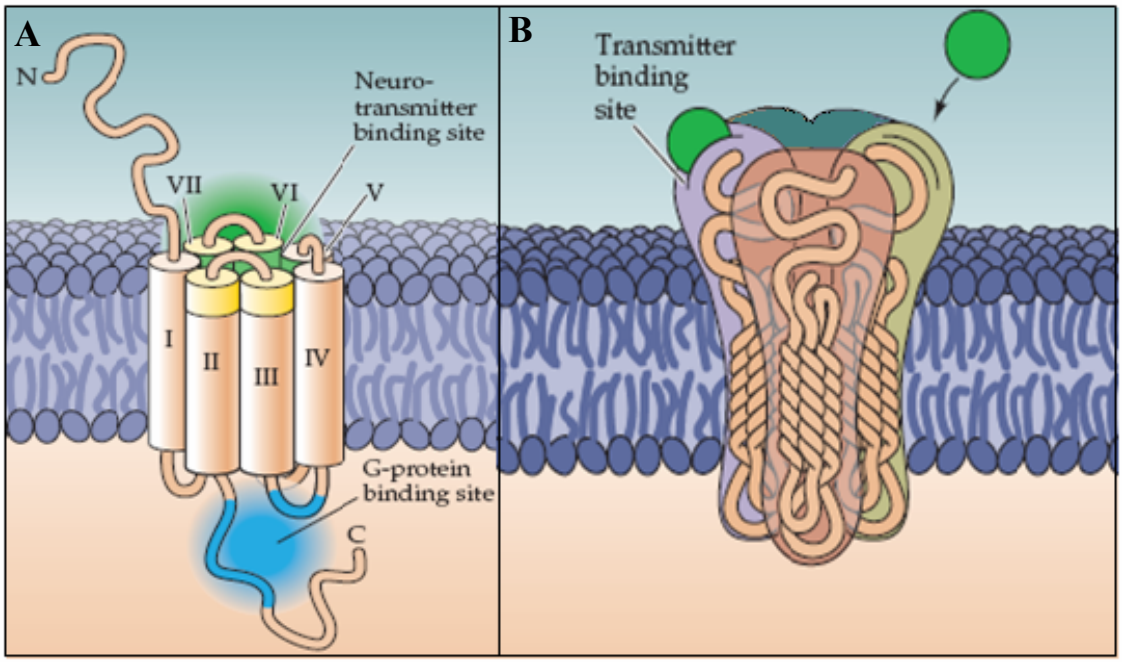

Figure 2 - The general structure of glutamate receptors. A – Metabotropic receptors; B – Ionotropic receptors (adapted from Purves, 2004).

1.2.2.1. Kainate receptors

Kainate receptors are homomeric or heteromeric complexes formed by the subunits GluR5–GluR7, KA1 and KA2 (Fig. 3A). Kainate receptors have similar fast activation and inactivation kinetics and a similar affinity for glutamate as the AMPA receptors (AMPAR). The agonists glutamate, kainate, quisqualate and domoate elicit currents with

inward rectification and low Ca^{2+} permeability (Lerma, 1997). The quinoxalinedione compounds, including 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX), are competitive antagonists of kainate receptors but exhibit very little selectivity between kainate and AMPA receptors. To solve this problem, several selective antagonists have been identified, such as (R,S)-3-(2-carboxybenzyl) willardiine (UBP296) and 5-carboxyl-2,4-di-benzamidobenzoic acid (NS3763) (Kew and Kemp, 2005).

Like AMPAR, kainate receptors can mediate excitatory synaptic signals, but they are also involved in modulation of neurotransmitter release, and therefore in regulating the strength of synaptic connections.

1.1.2.2. NMDA receptors

Ionotropic glutamate receptors with high affinity for the agonist NMDA are formed as heteromers that contain the NR1 subunit in combination with at least one type of NR2 subunits (Fig. 3B). The NR3 subunit does not form functional receptors alone, but can co-assemble with NR1/NR2 complexes (Cull-Candy et al., 2001). NMDA receptors have unique properties: their activation requires the simultaneous binding of two different agonists, glycine or D-serine, which bind to the NR1 subunits, and glutamate that binds to the NR2 subunits. NMDA receptors desensitise in the continued presence of the agonist. It has been shown that they exhibit different kinds of desensitisation mechanisms depending upon the glycine and Ca^{2+} concentrations (Mayer and Vyklicky, 1989; Sather et al., 1990).

These receptors are also characterized by voltage-dependent block by Mg^{2+} (Mayer et al., 1984; Nowak et al., 1984), high permeability to Ca^{2+} (MacDermott et al., 1986; Mayer and Westbrook, 1987), and slow gating kinetics (Lester et al., 1990; Ozawa et al., 1998). Furthermore, NMDA receptors have one or more modulatory sites that bind polyamines. Polyamines binding potentiate NMDA-mediated currents. However, at higher concentrations, polyamines generate a voltage dependent block of the ion channel, inhibiting receptor activation (Nestler, 2001).

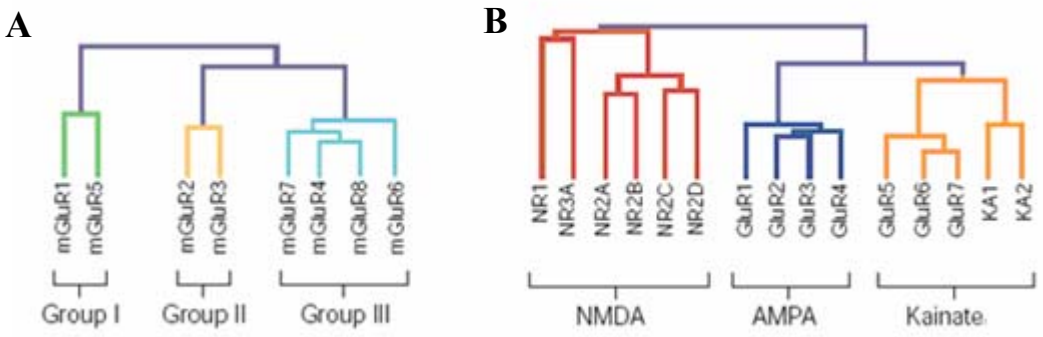

Figure 3 - Phylogenetic tree of glutamate receptor subunits.; A – Metabotropic glutamate receptors; B – Ionotropic glutamate receptors (adapted from Collingridge et al., 2004).

1.1.2.3. AMPA receptors

A third family of glutamate receptors is activated by AMPA. AMPAR are composed of subunits known as GluR1-4 or GluRA-D (Fig. 3B). These receptors are responsible for fast excitatory synaptic signaling and are involved in the activity-dependent modulation of synaptic strength. These changes in synaptic strength are involved in synaptic plasticity mechanisms, such as long term potentiation (LTP) and long term depression (LTD), which are thought to underlie learning and memory. When AMPAR are activated they allow Na^+ influx and K^+ efflux, but depending on subunit composition they can also be permeable to Ca^{2+} influx.

A large number of AMPAR agonists have been reported and one important aspect is that they can vary considerably in the amount of receptor desensitisation that they induce. Thus, whilst glutamate and AMPA act as full agonists and induce fast desensitisation, kainate acts as a partial agonist and induces slowly desensitising responses. Upon removal of agonist, receptors recover from desensitization and may be again activated. Likewise, many AMPAR antagonists have been described. However the most commonly used are CNQX and NBQX, which show a 20-150 fold selectivity for AMPA over kainate receptors (Kew and Kemp, 2005).

Since this work is focused exclusively on AMPAR, only their structure, molecular properties, trafficking regulation and expression, and not of kainate and NMDA receptors, will be described.

1.1.2.3.1. AMPA receptors structure

AMPAR are tetramers formed in the endoplasmic reticulum (ER) as dimers of dimers (Wu et al., 1996; Rosenmund et al., 1998; Mayer, 2006). These receptors are composed of structurally related subunits, four of which combine to form homomeric or heteromeric receptor complexes. The differences in the functional properties of AMPAR are consequence of different assemblies of these subunits. (Hollmann and Heinemann, 1994). The four subunits are structurally similar, sharing 68%-74% amino acid sequence identity and have a molecular weight of about 100 kDa (Jiang et al., 2006).

Each AMPAR subunit is composed by an extracellular N-terminal domain (NTD), three transmembrane domains (TM1, TM3 and TM4), one re-entrant transmembrane domain (TM2) that forms part of the channel pore, and an intracellular C-terminal domain (Fig. 4).

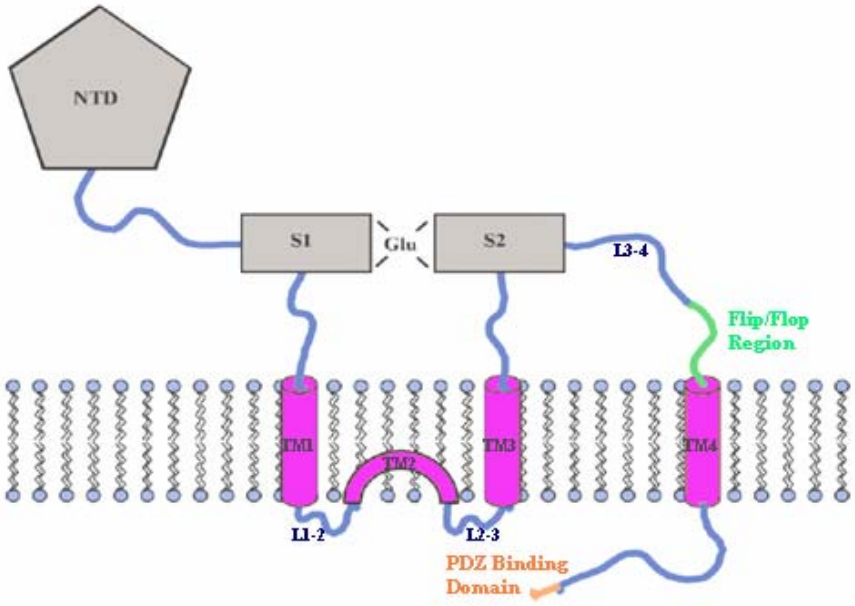

Figure 4 - Schematic representation of an AMPAR subunit. All subunits have similar structure and topology. They have three transmembrane spanning domains and the channel lining domain, between TM1 and TM3, is a re-entrant loop with both ends facing the cytoplasm (TM2). The transmembrane domains form two intracellular loops (L1–2 and L2–3) and one extracellular loop (L3–4). The glutamate binding site of AMPAR is formed by part of the N-terminal domain (NTD), S1, which together with a region of the extracellular loop (S2), form the glutamate binding site. The alternatively spliced flip/ flop region and the C-terminal PDZ binding domain are also shown (adapted from Bredt and Nicoll, 2003).

The transmembrane domains form two intracellular loops (L1–2 and L2–3) and one extracellular loop (L3–4). The third transmembrane domain leads to an extracellular loop

(S2) that, together with a part of the amino-terminal domain (S1), forms the glutamate binding domain (Fig. 4) (Jiang et al., 2006). Ligand linking to glutamate binding domain initiates conformational changes that are transduced to the transmembrane domains segments and promotes the opening of the channel's gate (Mayer, 2005).

AMPAR subunits have a considerable homology in their N-terminal domain and transmembrane domains. Intracellular C-terminal domain shows less homology between the subunits. However, it seems to have a high similarity among long forms and among shorter forms of AMPAR subunits. The GluR1, GluR4 and a splice variant of GluR2 (GluR2L) subunits have long cytoplasmic tails. In the opposite, GluR2 has a short tail similar to GluR3 and an alternative splice form of GluR4 (GluR4S). The cytoplasmic tail plays an important role in AMPAR trafficking and can interact with several intracellular proteins.

1.1.2.3.2. Post-transcriptional modifications

In addition to the variation created by the differential assembly of AMPAR subunits, some post-transcriptional modifications also contribute for AMPAR diversity, such as alternative splicing and ribonucleic acid (RNA) editing.

Splicing events influence the pharmacologic and kinetic properties of the channel. In all AMPAR, the extracellular loop (L3-4) is alternatively spliced as one of two distinct modules, flip and flop, that are respectively encoded by exons 14 and 15 (Fig. 4). The flop versions are generally less efficient because they desensitize much more rapidly than the flip forms (Sommer et al., 1990; Koike et al., 2000). This two forms display different expression profiles in the mature and in the developing brain. They are expressed at the same level in adult animals, but the flip form predominates before birth (Monyer et al., 1991). In addition, in the rat retina, it was also reported that the flop:flip ratio for GluR1 and GluR2 dramatically increases during postnatal development (Harada et al., 1998).

For GluR2 and GluR4 subunits, alternative splicing has also been described in the C-terminal domain. This event produces long and short isoforms, and influences the binding of specific interacting proteins, as well as the regulation of the receptors by protein phosphorylation (Song and Huganir, 2002).

RNA editing further increases the diversity of receptor subunits. Sequencing of the AMPA receptor subunit genes revealed that the arginine in the Q/R-site of GluR2 is not encoded genomically, but it is introduced by editing of a single nucleotide in the precursor messenger ribonucleic acid (mRNA)(Sommer et al., 1991). The positive charge and the size of the arginine residue prevent calcium (and other divalent cations) from permeating through the ion channel.

In addition to Ca^{2+} permeability, the Q/R site of GluR2 influences the single channel conductance properties of AMPAR and the sensitivity of the receptor to blockage by endogenous polyamines. AMPAR containing edited GluR2 show low channel conductance, and AMPAR lacking GluR2 subunit show high conductance (Swanson et al., 1997; Nestler, 2001).

1.1.2.3.3. Post-translational modifications

1.1.2.3.3.1 Phosphorylation

Phosphorylation of ligand-gated ion channels is crucial for the regulation of their function and plays an important role in synaptic plasticity and neuronal injury. AMPAR activity is partially regulated through phosphorylation and dephosphorylation by proteins kinases and phosphatases, respectively. All AMPAR subunits, with exception of GluR3, have been reported to be phosphorylated on several amino acid residues (Santos et al., 2008).

Phosphorylation of AMPA receptor subunits changes their activity, their surface expression and synaptic targeting, and influences their interaction with intracellular proteins. Cyclic-AMP-dependent protein kinase (PKA) has an important role in the insertion of GluR1 (by phosphorylation of Ser845) and GluR4 (phosphorylation of Ser842) subunits into synapse (Roche et al., 1996; Esteban et al., 2003). Protein kinase C (PKC) is involved in the delivery of GluR1-containing AMPARs to the synapse and/or in their stabilization in the synaptic compartment (phosphorylation at Ser818) (Boehm et al., 2006). Calcium/calmodulin-dependent protein kinase II (CaMKII), a major constituent of postsynaptic density at glutamatergic synapses, and PKC phosphorylate GluR1 at Ser831, increasing the single channel conductance (Barria et al., 1997; Mammen et al., 1997;

Hayashi et al., 2000). Furthermore, PKC phosphorylation of GluR2 at Ser880 promotes receptor internalization and regulates interaction of this subunit with glutamate receptor-interacting protein (GRIP) and protein interacting with C kinase 1 (PICK1) (Matsuda et al., 1999; Chung et al., 2000; Perez et al., 2001).

1.1.2.3.3.2. Palmitoylation

Protein palmitoylation is a common protein modification in which a 16-carbon-atom saturated fatty acid (palmitate) is covalently attached to a cystein residue through a thioester bond. All four types of AMPA receptor subunits are palmitoylated at two sites on their C-terminal and transmembrane re-entrant domains (Hayashi et al., 2005).

Several evidences suggest that palmitoylation has an important role in the postsynaptic trafficking of AMPAR and in the regulation of excitatory synaptic transmission. Palmitoylation of transmembrane re-entrant domain causes accumulation of the receptor in the Golgi apparatus (Hayashi et al., 2005). In addition, palmitoylation on the C-terminal domain inhibits its interaction with the 4.1N protein, which has previously been shown to stabilize AMPA receptor expression at the cell surface (Shen et al., 2000; Hayashi et al., 2005).

1.1.2.3.3.3. Glycosylation

Protein glycosylation is the transfer of glycosyl groups to side chain groups of asparagine, serine and threonine. If glycosylation occurs on asparagine it is called N-linked.

All AMPAR subunits are *N*-glycosylated at multiple sites on their extracellular domains. This modification has an important role in AMPAR maturation, protects from proteolytic degradation, and affects receptors' current amplitudes (Jiang et al., 2006).

1.1.2.3.4. AMPA receptors trafficking

As AMPAR are the major determinant of post-synaptic excitability, the density of these receptors must be carefully regulated. AMPAR reach their synaptic targets after an extremely complicated intracellular trafficking pathway in which almost every step is under of a tight control.

1.1.2.3.4.1. Assembly and exit from the endoplasmic reticulum

The subunit stoichiometry of AMPA receptor tetramers is crucial for synaptic function. These oligomeric combinations are formed in the rough endoplasmic reticulum (ER) and inserted into the plasma membrane after crossing the Golgi apparatus. After assembly, exit from the ER is tightly regulated by quality control mechanisms that monitor the competency of newly synthesized receptors for ligand binding and gating (Mah et al., 2005).

AMPAR subunits are assembled in the ER by successive formation of dimers and tetramers. The initial stage is the dimerization via interactions between the N-terminal domains of the subunits (Leuschner and Hoch, 1999; Ayalon and Stern-Bach, 2001). This is followed by a second dimerization (also called tetramerization) that is mediated by associations at the ligand binding and membrane domains, which are then stabilized by secondary N-terminal domain interactions (Ayalon et al., 2005; Greger et al., 2006). This step is also dependent on Q/R editing of GluR2 subunits since edited R subunits are largely unassembled and ER retained, whereas unedited Q subunits readily tetramerize and traffic to synapses (Greger et al., 2003). The retention protein that prevents immature GluR2 from exiting the ER is not well known; however, ER chaperones BiP and calnexin associate with a fraction of AMPAR and may play a role in the retention mechanism (Rubio and Wenthold, 1999; Greger et al., 2002). Additionally, researchers have identified proteins that are involved in the regulation of AMPAR export from ER and that interact with C-terminal domain of these receptors. For example, PICK 1, a PDZ domain-containing protein, binds to a PDZ consensus motif (SVKI) of GluR2, and this interaction may be necessary for the exit of GluR2 from ER (Greger et al., 2002).

Transmembrane AMPAR regulatory proteins (TARPs) are considered auxiliary proteins for AMPAR. These proteins interact with all AMPAR subunits controlling their maturation and trafficking (Fukata et al., 2005; Nicoll et al., 2006; Ziff, 2007). Stargazin is the founding member of TARPs and has an important role in AMPA receptor trafficking (Chen et al., 2000; Vandenberghe et al., 2005b). The actions of this protein start at the early stages of receptor biogenesis, contributing to the folding and assembly of AMPAR and facilitating their export from the ER to the Golgi Complex (Fig. 5) (Vandenberghe et al., 2005a; Bedoukian et al., 2006).

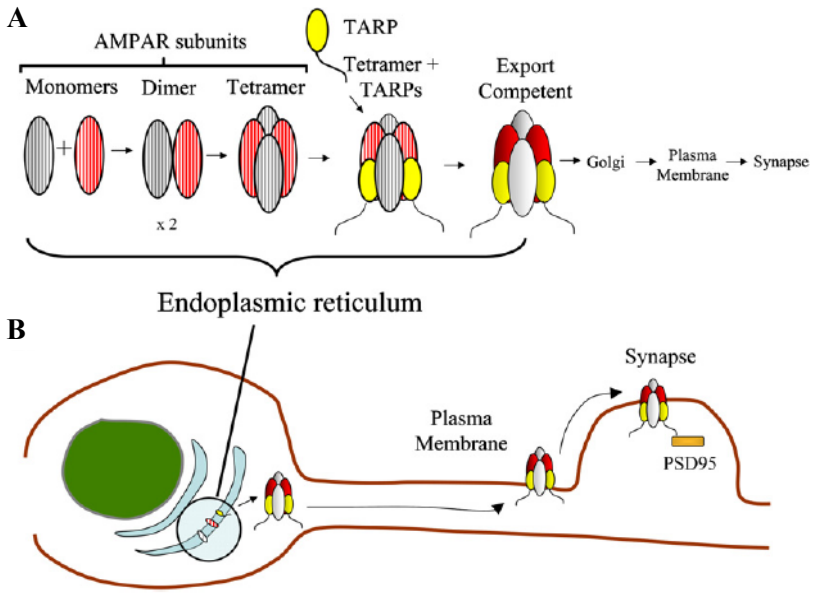

Figure 5 - A – AMPAR are assembled in the endoplasmic reticulum (ER) by successive formation of dimers and tetramers. Transmembrane regulatory proteins (TARPs) associate with AMPAR and generate an ER export-competent receptor; B – This complex exits from ER and traffics to the plasma membrane where it is inserted at an extrasynaptic site. The complex diffuses to the synapse, where TARP C-terminal PDZ binding site interacts with the protein PSD-95 promoting the anchorage of the complex at the synapse (adapted from Ziff, 2007).

1.1.2.3.4.2. Dendritic trafficking

The trafficking of AMPAR along dendrites is dependent on the microtubular cytoskeleton, and their transport is an active process involving motor proteins such as dynein and kinesin (Hirokawa and Takemura, 2005). For example, glutamate receptor-interacting protein/AMPA binding protein (GRIP/ABP) acts like an adaptor to link AMPAR to kinesines, promoting their transport. The multiple PDZ domain-containing protein GRIP/ABP interacts directly with the heavy chain of conventional kinesin (KIF5)

(Setou et al., 2002) and binds to the C-terminal PDZ motif of GluR2 and GluR3 (Dong et al., 1997).

AMPAR that are traveling along dendritic microtubules must be transferred to dendritic spines to access the synapse. Dendritic spines are small protrusions along dendrites that form small microcompartments and are enriched with high motile actin filaments. Therefore, there are proteins that promote the link between actin filaments and AMPAR. The neuronal isoform of 4.1N interacts with GluR1 (Shen et al., 2000) and GluR4 (Coleman et al., 2003), and appears to stabilize their surface expression, whereas RIL (reversion induced LIM protein), that has a PDZ domain, binds to GluR1 and promotes the transport of AMPAR into dendritic spines (Schulz et al., 2004).

Another interesting fact is that different subunits have different trafficking dynamics that are independent of neuronal activity. It was suggested that GluR1 is constitutively and rapidly transported throughout the neuron, whereas GluR2 is less mobile and mostly retained in a relatively immobile membrane-associated clusters (Perestenko and Henley, 2003).

1.1.2.3.4.3. Membrane insertion and stabilization

The final step of insertion and stabilization of AMPAR into the specialized dendritic membrane that constitutes the postsynaptic terminal involves tightly regulated events that are dependent on the subunit composition. Therefore, postsynaptic densities are enriched with accessory and scaffolding proteins, which have been shown to participate in this process.

Postsynaptic density-95 protein (PSD-95) and other members of the PSD-95 family are critical determinants for synaptic targeting of AMPAR (Chen et al., 2000; Tomita et al., 2005; Beique et al., 2006; Elias et al., 2006). Although PSD-95 does not directly link to the receptors, it binds to proteins of TARP family, which in turn interact with AMPAR subunits (Fig. 5).

PICK1 has a PDZ domain through which it can bind to the C-terminal domain of GluR2 subunit. PICK1 stabilizes and binds to intracellular pools of GluR2 (Steinberg et al., 2004; Gardner et al., 2005; Liu and Cull-Candy, 2005) and has also an important role in receptor internalization (Iwakura et al., 2001; Perez et al., 2001).

Insertion of AMPAR into the membrane is mediated by N-ethylmaleimide-sensitive factor (NSF) that interacts with C-terminal domain of GluR2 (Nishimune et al., 1998; Osten et al., 1998; Song et al., 1998) regulating the rapid endocytosis of this subunit at synaptic sites (Beretta et al., 2005). Moreover, NSF is involved in the regulation of the interaction between GluR2 and PICK1 (Hanley et al., 2002). Another family of AMPAR interacting proteins, the neuronal pentraxins, NARP, NP1 and NPR, are involved in synaptic retention of AMPAR (Song and Huganir, 2002), probably because they reduce the lateral diffusion and endocytosis of these receptors (Shepherd and Huganir, 2007).

The mechanisms regulating AMPA receptor exocytosis are governed by subunit-specific rules. GluR2-GluR3 heteromers continuously cycle in and out of synapses in a manner independent of synaptic activity. This constitutive cycling is rapid and preserves the total number of receptors at synapses. In contrast, exocytosis of AMPAR containing GluR1, GluR2 long (a splice isoform of GluR2) or GluR4 is slow and activity-dependent during synaptic plasticity (Hayashi et al., 2000; Zhu et al., 2000; Kolleker et al., 2003).

Increasing evidence indicate that the transport of AMPAR in and out of the synaptic membrane is essential to activity-dependent changes in synaptic strength. The importance of synaptic plasticity and the mechanisms of LTP and LTD in AMPAR trafficking and surface expression will be discussed in section 3.2.

1.1.2.3.4.4. Endocytosis and recycling

The controlled endocytic removal of AMPAR from synapses is as critical for synaptic function as the delivery of new receptors. Clathrin-mediated endocytosis has a central role in AMPA receptor internalization (Hirling, 2008). Clathrin adapters are essential for endocytosis by linking membrane proteins to clathrin and facilitating the assembly of clathrin coats. The protein AP2 is the best characterized member of clathrin adaptor complexes. This protein interacts with GluR2 subunit and plays an important role in the vesicle trafficking pathway (Jiang et al., 2006).

After internalization, AMPAR can be taken by early endossomes to a recycling endossome compartment that allows quick reinsertion in the dendritic surface, or by late endossomes and lysossomes that allow degradation of the receptors (Ehlers, 2000; Lee et al., 2004).

2. Retina

The retina is the light sensitive inner layer of the eye. This layer is responsible for converting images of the external environment into neural impulses, and transmits them through the optic nerve to the brain for decoding and analysis. Despite its peripheral location, the retina is part of the CNS.

The retina is constituted by several layers. The retinal pigment epithelium (RPE) is a densely packed single layer of cells, with pigment granules, that is located between the photoreceptors and Bruch's membrane. The next three layers are composed by the different regions of photoreceptors: the photoreceptors outer segments (that are in contact with RPE), the outer limiting membrane (site of occluding junctions between the plasma membranes of photoreceptors and Müller cells), and the outer nuclear layer (constituted by the photoreceptors nuclei). The region containing synapses between the photoreceptors and bipolar or horizontal cell dendrites is known as the outer plexiform layer. The next layer, the inner nuclear layer, is composed by cell bodies of horizontal, bipolar, amacrine and Müller cells, and the inner plexiform layer is where the bipolar and amacrine cells connect to the ganglion cells. Ganglion cells layer is composed by the nuclei of ganglion cells and some amacrine displaced cells. The ganglion cells axons bundle together and form the nerve fiber layer (Fig. 6). Finally, the inner limiting membrane is formed by the end feet of Müller cells and is the limit between the retina and the vitreous body (Forrester et al., 2002).

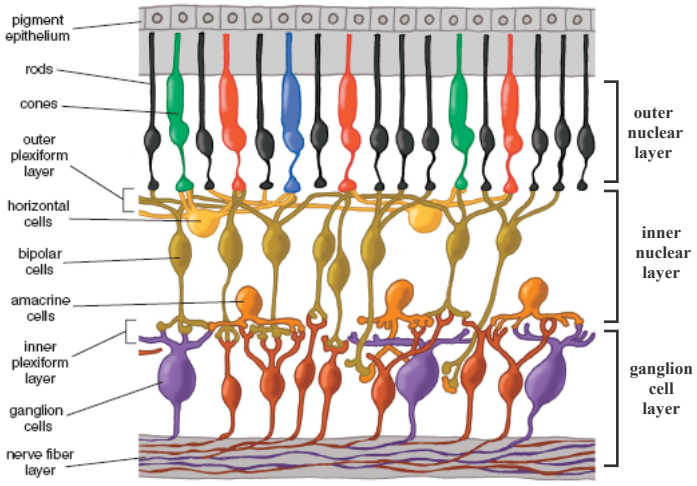

Figure 6 - Schematic representation of retinal cell layers (adapted from Kolb, 2003).

The retina is composed by different types of cells from which neural cells predominate. However, there are also glial cells, vascular endothelium and perycites.

The photoreceptors are responsible for the absorption of light and initiation of the neuron-electrical impulse, and they can be classified as rods or cones. Rods are responsible for non-chromatic vision and are sensitive to very dim light, whereas cones deal with bright signals and can detect rapid light fluctuations. Both photoreceptors contain light sensitive pigments, rods have rodopsin, and cones have three types of opsins. They also differ in shape: rods are long and slender and cones are short and tapered. In addition, the distribution of each type of photoreceptor is not similar. The fovea is a retinal depression located in the center of the retina. This region does not contain rods, but contains the highest density of cones, which allows high acuity.

Bipolar cells are primarily responsible for transmitting signals from photoreceptors or horizontal cells to ganglion or amacrine cells.

Ganglion cells interact with amacrine and bipolar cells, their axons form the nerve fiber layer, and make synapses with cells in the lateral geniculate nucleus of the thalamus. There are different types of ganglion cells, which are classified based on cell body, dendritic tree spread and branching pattern.

Horizontal cells are mainly inhibitory neurons that process the information from a large number of photoreceptors and synapse onto bipolar cells, in the outer plexiform layer of the retina.

Amacrine cells are interneurons that are located in the inner nuclear layer. These cells play a role in modulation, mostly inhibitory, of the signals reaching ganglion cells.

Astrocytes are predominantly located in the ganglion cell layer, inner plexiform layer and inner nuclear layer. Although astrocytes contact with neurons, they are wrapping retinal blood vessels. They have an important role in preventing unwanted signals from the surrounding neurons because they isolate the receptive surfaces of neural cells. Astrocytes contain also abundant glycogen and they may form a nutritive service in providing glucose to the neurons.

Müller cells are the principal supporting glial cells of the retina. Their cell bodies are located in the inner nuclear layer and project irregularly thick and thin processes in either direction to the outer limiting membrane and to the inner limiting membrane. Müller cells have an important role in retina homeostasis, supporting and nourishing neural cells.

They are also implicated in the modulation of neuronal activity by controlling the neuronal microenvironment and the concentration of neuroactive substances.

Microglial cells are ubiquitously distributed in every layer of the retina. These cells become activated upon injury and assume the role of wandering phagocytes (Forrester et al., 2002).

2.1. Visual pathway

Retina takes light energy and transforms it into electrical signals. Light must pass through the first layers before reaching the photoreceptors. The light is absorbed by the rods and cones photopigments located in the outer segments. RPE absorb the stray light, preventing the reflection and scattering of light within the eyeball. Through a mechanism known as phototransduction, light energy is converted into electrical impulses that pass along the visual pathway. Impulses are passed to bipolar neurons that in turn contact ganglion cells that propagate an action potential down its axons to the brain. This process is called the vertical transduction pathway in the retina, and the principal neurotransmitter involved is glutamate. The vertical pathway is modulated by lateral inputs from horizontal cells that mediate receptive field organization of bipolar cells and make connections between the photoreceptors, and from amacrine cells that interact with bipolar and ganglion cells in the inner plexiform layer. Horizontal and amacrine cells generally release the neurotransmitters gamma-aminobutyric acid (GABA) or glycine to modulate the vertical signal flow. This pathway, mediated by horizontal and amacrine cells, is called lateral pathway (Purves, 2004).

2.2. Glutamate and glutamate receptors in the retina

In the retina, differing from most sensory systems, photoreceptors do not exhibit action potentials. Light processing within the retina is mediated by graded potentials, mostly because action potentials are not necessary to transmit information over the relatively short distances involved. Only ganglion cells propagate action potentials down its axons to the brain. The action potential is necessary at this point because the signal must be carried for a longer distance.

Another special feature of the retina is the selective response of ON and OFF bipolar cells to light. In the dark, photoreceptors depolarize and release glutamate tonically. In ON bipolar cells, glutamate binds to metabotropic glutamate receptors (mGluR6) and activates intracellular cascades that close cGMP-gated Na^+ channels, and consequently hyperpolarize these cells. On the contrary, OFF bipolar cells, that express AMPA and kainate receptors, depolarize in response to glutamate released from photoreceptors. In contrast to the dark situation, light stimulation hyperpolarizes photoreceptors, decreasing the release of neurotransmitter. Consequently, ON bipolar cells are not under the hyperpolarizing influence of glutamate and depolarize. However, in OFF bipolar cells, the reduction in neurotransmitter represents the withdrawal of a depolarizing influence and these cells hyperpolarize (Purves, 2004).

Glutamate is also released by bipolar cells to transmit the ON and OFF signals to amacrine and ganglion cells in the inner plexiform layer, and by ganglion cells that transmit the visual information to the brain (Marc et al., 1990; Marc et al., 1995).

Glutamate receptors are present in several types of cells within the retina. The differential expression of neurotransmitter receptors is the basis of the multiple processing of visual signals.

Immunocytochemistry studies and hybridization *in situ* revealed the presence of all mGluRs in the retina. These receptors have a heterogeneous and widespread expression, particularly in the outer plexiform layer, inner nuclear layer, inner plexiform layer, and the ganglion cell layer. Also, it was demonstrated an involvement of mGluRs in both presynaptic and postsynaptic glutamatergic signaling (Gerber, 2003).

Ionotropic GluRs account for most of the fast excitatory synaptic transmission in the CNS. NMDA receptors were first identified in the retinal cells by *in situ* hybridization (Brandstatter et al., 1994). Later, immunocytochemistry experiments revealed the presence of several NMDA receptor subunits in different neuronal retinal cell types (Brandstatter et al., 1998; Thoreson and Witkovsky, 1999; Fletcher et al., 2000).

As for NMDA receptors, kainate receptors subunits were initially studied at the level of the receptor transcripts (Brandstatter et al., 1994). Subsequently, immunocytochemical studies verified the localization of several kainate receptors subunits in horizontal, amacrine, ganglion and bipolar cells (Peng et al., 1995; Brandstatter et al., 1997; Brandstatter et al., 1998).

In situ hybridization studies also demonstrated a differential and widespread expression of AMPAR subunits mRNA in the mammalian retina. Later, immunocytochemical studies reported the presence of AMPAR subunits in all cell layers of the vertebrate retina (Peng et al., 1995). In the rat retina, GluR2 is the most abundant subunit, followed by GluR1, GluR3 and GluR4 (Kamphuis et al., 2003).

In rat, the immunoreactivity of GluR1 is present mostly in the inner plexiform layer and in certain types of amacrine cells in the inner nuclear layer (Peng et al., 1995). GluR1 immunostaining was also found in the ganglion cell layer, in AII amacrine cells and cholinergic cells in postnatal rat retina (Grunder et al., 2000).

The staining pattern of GluR2 and GluR3 is difficult to distinguish because there are no good antibodies selective for GluR3 subunits. In cat, GluR2/3 expression was detected in horizontal cells, bipolar cells, different populations of amacrine cells and ganglion cells (Kolleker et al., 2003). In rat, GluR2/3 subunits are present in inner and outer plexiform layers, in ganglion cell layer and in some bipolar and amacrine cell bodies (Peng et al., 1995).

GluR4 expression in the outer part of the inner nuclear layer and in the outer plexiform layer in the mammalian retina was demonstrated by *in situ* hybridization (Hamassaki-Britto et al., 1993) and immunohistochemistry (Peng et al., 1995; Kolleker et al., 2003). This subunit was also found in Müller cells (Peng et al., 1995).

3. Hippocampus

The hippocampus is one of the brain structures that belong to the limbic system. The hippocampus is typically described as a trisynaptic circuit or loop that is composed by three subfields, area CA1 and area CA3, both composed mainly of pyramidal cells, and the *dentate gyrus*, where the principal type of cell is the granule cell (Fig. 7).

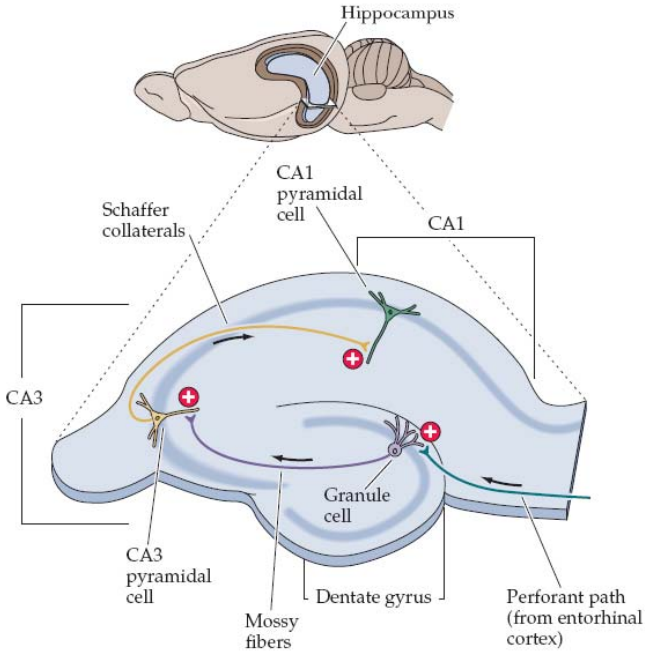

Figure 7 - Diagram of a sagittal section of a rodent hippocampus showing the major regions, excitatory pathways, and synaptic connections (Adapted from Purves, 2004).

This trisynaptic circuit is composed of three sequential glutamatergic synapses. The afferents inputs are carried by perforant path axons of layer II of entorhinal cortex to the *dentate gyrus*. Granule cells in the dentate gyrus project their axons (mossy fibers) to the apical dendrites of CA3 pyramidal cells, which, in turn, project their Schaffer collateral axons to apical dendrites of pyramidal cells in area CA1. CA1 axons project to subiculum that send information along the output pathways of the hippocampus (Fig. 7) (Purves, 2004).

3.1. Glutamate receptors in the hippocampus

Glutamate receptors are found in almost all neurons and in many types of glia in the brain, and each type of ionotropic glutamate receptor subunit shows a distinct pattern of expression.

NMDA receptors are found throughout the brain, but predominantly within the forebrain. However, the highest levels in the brain are found in the CA1 region of the hippocampus, where they have an important role in synaptic plasticity (Ozawa et al., 1998).

Kainate receptor subunits are differentially distributed in the CNS. Whereas KA2, GluR5 and GluR7 transcripts are widely expressed in the brain, KA1 was found mainly in the CA3 region and *dentate gyrus* of the hippocampus and GluR6 mRNA is most abundant in cerebellar granule cells (Ozawa et al., 1998).

Regarding AMPAR subunits, GluR1, GluR2 and GluR3 mRNAs are abundantly expressed in the pyramidal cell layer and *dentate gyrus*. AMPAR subunits are also differentially expressed during development. In mature mammalian hippocampus heteromers of GluR1-GluR2 and GluR2-GluR3 are predominantly found (Ozawa et al., 1998), whereas GluR4 is expressed mainly in early postnatal development (Zhu et al., 2000).

3.2. Synaptic plasticity

Synaptic plasticity is the property of synapses to change their characteristics. Many forms of synaptic plasticity have been discovered in the mammalian CNS, but LTP and LTD of excitatory synaptic responses in hippocampus have been the most extensively studied (Nestler, 2001). LTP is a persistent increase in the efficacy of synaptic transmission, following a short period of high-frequency synaptic stimulation, whereas LTD is characterized by a reduction in synaptic strength after a period of low-frequency synaptic stimulation (Malenka and Bear, 2004). The bidirectional control of synaptic strength by these mechanisms is believed to underlie some forms of learning and memory in the mammalian brain (Nestler, 2001).

LTP is induced by high-frequency stimulation, which causes a prolonged depolarization resulting in Mg^{2+} removal from the NMDA channel. Therefore, NMDA activation promotes an increase in Ca^{2+} concentration within the dendritic spines of the postsynaptic cell, which in turn triggers the LTP. The Ca^{2+} ions activate postsynaptic protein kinases. These kinases are responsible for delivery of new AMPAR into the postsynaptic spine, thereby increasing the sensitivity to glutamate (Fig. 8A).

LTD occurs when neurons are stimulated at a low rate for long periods. This stimulus also activates NMDA receptors, promoting a low-amplitude rise in Ca^{2+} concentration in postsynaptic neurons, which activates protein phosphatases. Phosphatases cause internalization of postsynaptic AMPAR, decreasing the sensitivity to glutamate released from presynaptic terminals (Fig. 8B).

Thus, the rise of Ca^{2+} (intracellular) determines the presence of LTP or LTD: small increase in Ca^{2+} leads to depression, whereas a large increase triggers potentiation (Purves, 2004).

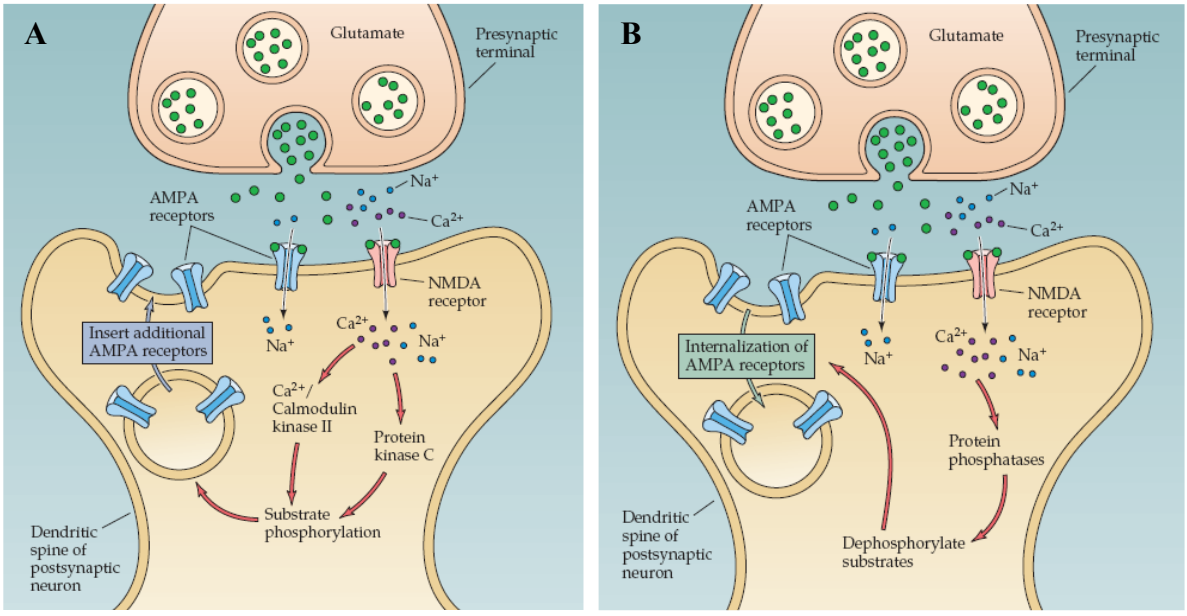

Figure 8 - Mechanisms underlying LTP and LTD. A – LTP increases AMPA receptor delivery to the membrane and stabilization at the synapse, through a calcium-driven process that involves CAMKII and fusion of recycling endosomes; B – Induction of LTD enhances AMPAR endocytosis at postsynaptic membrane. This process is calcium-dependent and involves protein phosphatases (Adapted from Purves, 2004).

4. Diabetes *mellitus*

Diabetes *mellitus* is a metabolic disorder characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism. The increase in the concentration of glucose in the blood results from defects of insulin secretion, action or both. There are three main types of diabetes: type 1, which is characterized by a decrease or a lack of insulin production due to lesions in pancreatic β cells; type 2, that is most often associated with older age, obesity or family history and is caused by the body's ineffective use of insulin (insulin resistance), combined with reduced insulin secretion; and gestational, that woman develops during pregnancy and usually do not prevail after the child birth.

Diabetes is a serious problem of public health. In 2000, all over the world, there were about 171 millions of cases of diabetes among people over 20 years of age, and it was estimated that this number will increase up to 366 millions in the year 2030 (Wild et al., 2004).

4.1. Diabetic retinopathy

Diabetic retinopathy is the most common ocular complication of diabetes *mellitus* and is one of the leading causes of vision loss and blindness in developed countries (VanNewkirk et al., 2001; Kempen et al., 2004).

Diabetic retinopathy has been considered a disease of the retinal vasculature, since vessel integrity becomes increasingly compromised. According to the classification described by Cunha-Vaz, diabetic retinopathy is defined in five stages: 1) Pre-clinic retinopathy is characterized by the absence of ophthalmoscopic lesions. However, diagnostic techniques more sensitive show endothelial proliferation in capillaries and venules, and edema and endothelial degeneration in arterioles; 2) In nonproliferative diabetic retinopathy it becomes possible to detect microaneurysms, intraretinal hemorrhages, hard exudates and retinal edemas; 3) The macular edema is an accumulation of fluid in the macula causing swelling, which disturbs central vision; 4) The preproliferative stage is characterized by the increase of ischemic lesions in the retina, such as cotton-wool exudates; 5) Finally, in proliferative retinopathy it is observed the

formation of neovessels (neovascularization), which form areas without capillary perfusion (Cunha-Vaz, 2006).

4.1.1. Neuronal dysfunction in diabetic retinopathy

In addition to the vascular lesions, there are functional and morphological alterations in retinal neurons and glia during diabetes, and these changes may occur before the onset of visible vascular disease (Ewing et al., 1998; Lieth et al., 2000; Ozdek et al., 2002).

Dysfunction of retinal neurons during diabetes is probably the cause of loss of contrast discrimination and color vision in patients with diabetic retinopathy (Roy et al., 1986; Purves, 2004). Furthermore, changes in electroretinogram (ERG) were reported in diabetic patients and rats (Sakai et al., 1995; Fortune et al., 1999; Hancock and Kraft, 2004; Kohzaki et al., 2008). An ERG is an electrophysiological test that measures changes in field potentials generated by retinal neurons. In a recent study it was suggested that impaired local responses on multifocal electroretinograms predict subsequent development of vascular disease (Han et al., 2004).

Several evidences indicate that there is an increase in apoptosis in the retina. Early studies described the existence of pyknosis in neuronal cell bodies in histological sections of the retina from diabetic people (Wolter, 1961; Bloodworth, 1962). More recently, apoptosis was identified in sections of retinas from streptozotocin-induced diabetic rats and in retinal cell cultures exposed to high glucose, by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) staining (Hammes et al., 1995; Santiago et al., 2007).

It has been suggested that glutamate, the principal excitatory neurotransmitter in the retina, could be related with the development of diabetic retinopathy (Pulido et al., 2007). It is widely established that overactivation of ionotropic glutamate receptors cause excitotoxicity. Indeed, glutamate concentration is elevated in the vitreous of patients with proliferative diabetic retinopathy (Ambati et al., 1997). As referred previously, glial cells have an important role in glutamate-glutamine cycle. In diabetic rats, retinal glial cells have reduced ability to convert glutamate into glutamine (Lieth et al., 1998). Furthermore, it was demonstrated that high glucose and diabetes increase the release of D-aspartate (a

non-metabolizing analogue of glutamate) evoked by membrane depolarization in retinal neural cell cultures and in the retina, respectively (Santiago et al., 2006a).

Recently, in our laboratory, Santiago and colleagues found that high glucose alters the content of ionotropic glutamate receptor subunits in cultured rat retinal neurons, since it decreases GluR1 and GluR6/7 protein levels, and increases GluR2 and KA2 levels (Santiago et al., 2006b). Furthermore, these authors reported an increase in the levels of GluR2 and NR1 subunits in the post-mortem retinas of diabetic patients, namely in the inner and outer plexiform layers (Santiago et al., 2008).

In addition, the activation of PKC has been implicated in the pathogenesis of diabetic retinopathy (Curtis and Scholfield, 2004). This evidence suggests that the phosphorylation levels of AMPAR may be altered and consequently AMPAR trafficking to and from the cell membrane might be affected.

Altogether these changes may have a direct impact in retinal neurotransmission that may lead to visual field defects. However, the evidences supporting this hypothesis are still scarce.

4.2. Cognitive dysfunction in diabetes

Diabetes *mellitus* has been associated with moderate impairment in cognitive function (Biessels et al., 1994; Stewart and Liolitsa, 1999). This cognitive impairment is characterized by lowered performance on several cognitive domains, particularly slowing mental speed and diminishing mental flexibility (Dejgaard et al., 1991). Likewise, in animal models, behavioral studies performed in streptozotocin-induced diabetic rats, such as shock avoidance and spatial water maze learning, reported impairments in hippocampal-mediated memory processes (Biessels et al., 1996).

Memory and LTP impairments in diabetic rats might be due to abnormalities in postsynaptic glutamatergic receptors. LTP magnitude generated after high-frequency stimulation was reduced in hippocampal slices from diabetic rats (Biessels et al., 1996). Furthermore, the activation and modulation of AMPAR may be impaired in streptozotocin induced diabetes. It was reported that [^{3}H]AMPA binding is reduced in several brain regions, including hippocampus (Chabot et al., 1997).

It was also demonstrated that hippocampal NMDA receptor expression in diabetic rats is altered (Gardoni et al., 2002). However, until now, there are no evidences about the effect of diabetes on AMPAR surface expression, and the importance of these potential changes for the development of cognitive dysfunction in diabetes is still unknown.

5. Objectives

Diabetic retinopathy and cognitive and memory impairments are common complications of diabetes *mellitus*, and hyperglycemia is considered the main factor for the development of diabetes-associated pathologies. Some reports have demonstrated that diabetes or elevated glucose can alter the content of different ionotropic glutamate receptor subunits in the retina and brain, including hippocampus (Gardoni et al., 2002; Ng et al., 2004; Santiago et al., 2006b; Santiago et al., 2008). These alterations may affect glutamatergic neurotransmission in the retina and hippocampus.

In a previous report, from our laboratory, Santiago and colleagues have demonstrated, although indirectly, that elevated glucose (7 days exposure) increases the content of GluR2 subunit of AMPA receptors at the plasma membrane, since the number of retinal cells with Ca^{2+}-impermeable AMPA receptors present at the plasma membrane, which possess GluR2 subunits, increased, as shown by cobalt staining technique and Ca^{2+} imaging experiments (Santiago et al., 2006b). Here, our main goal was to further investigate, using a biotinylation assay, whether elevated glucose could affect the content of different AMPA receptors subunits at the level of plasma membrane in retinal cells, and also in hippocampal cells. For this purpose, we assessed both total and surface protein content in cell cultures exposed to high glucose (to mimic hyperglycemia).

III - Materials and Methods

1. Materials

Fetal bovine serum, trypsin (GIBCO), Neurobasal Medium (GIBCO), B27 supplement (GIBCO), secondary antibodies Alexa 488 goat anti-rabbit and Alexa 568 goat anti-mouse (Molecular Probes), and primary antibody anti-transferrin receptor were purchased from Invitrogen Corporation (Carlsbad, CA, USA). Penicillin, streptomycin**,** DAPI **(**4',6-diamidino-2-phenylindole), goat serum, poly-D-lysine hydrobromide, D-glucose and D-mannitol were purchased from Sigma-Aldrich Corporation (St. Louis, Missouri, USA). Protease inhibitor cocktail tablets *complete-mini* were purchased from Roche (Basel, Switzerland). Sodium sodecyl sulfate (SDS) and polyacrilamide were acquired from BioRad (Richmond, Virginia, USA). ECF (enhanced chemifluorescence) substrate, primary anti-actin antibody and anti-mouse and anti-rabbit alkaline phosphatase-conjugated secondary antibodies were purchased from GE Healthcare (Chalfont St. Giles, UK). Polyvinylidene fluoride (PVDF) membranes and the primary antibodies anti-GluR1, -GluR2 and -GluR4 were obtained from Millipore (Billerica, Massachusetts, USA). BCA (bicinchoninic acid), Protein Assay Kit, NeutrAvidin agarose resin and Sulfo-NHS-SS-Biotin were purchased from Pierce, Thermo Scientific (Rockford, Illinois, USA). All other reagents were acquired from Sigma-Aldrich Corporation (St. Louis, Missouri, USA) or BioRad (Richmond, Virginia, USA).

2. Animals

The animals were handled in agreement with the EU guidelines (86/609/EEC) for the protection of animals used for experimental and other scientific purposes. All efforts were made to minimize the suffering and the number of animals used.

3. Cell cultures

3.1. Primary retinal neural cell cultures

Primary retinal neural cell cultures were prepared from 3- to 5-days-old Wistar rats. The retinas were dissected in sterile Ca^{2+} - and Mg^{2+}- free Hanks' balanced salt solution (HBSS) (137 mM NaCl, 5.4 mM KCl, 0.45 mM KH_2PO_4, 0.34 mM Na_2HPO_4, 4 mM $NaHCO_3$, 5 mM Glucose, pH 7.4) and digested with 0.1% trypsin for 15 min at 37ºC. After dissociation, the cells were centrifuged (1 min, 1000 rpm) and resuspended in Eagle's minimum essential medium (MEM), containing 5.5 mM glucose, buffered with 26 mM $NaHCO_3$ and 25 mM HEPES, and supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 μg/mL) (pH 7.4). The cells were cultured at a density of 2×10^6cells/cm^2 on poly-D-lysine coated plates. The cells were maintained at 37ºC for 7 days in vitro (DIV) in a humidified incubator with 5% CO_2 and 95% air.

3.2. Primary cultures of hippocampal neurons

Primary cultures of rat hippocampal neurons were prepared from Wistar rat embryos (E18–E19). The hippocampi were dissected and then digested with 0.06% trypsin for 15 min at 37ºC in HBSS containing: 137 mM NaCl, 5.36 mM KCl, 0.44 mM KH_2PO_4, 0.34 mM $Na_2HPO_4.2H_2O$, 4.16 mM $NaHCO_3$, 5 mM glucose, 1 mM sodium pyruvate, 10 mM HEPES and 0.001% phenol red; pH 7.4. After digestion, the hippocampi were washed in HBSS supplemented with 10% fetal bovine serum, to stop trypsin action, and mechanically dissociated. Hippocampal cells were then transferred to Neurobasal medium, supplemented with B27 supplement (1:50 dilution), 25 μM glutamate, 0.5 mM glutamine and 0.12 μg/ml gentamicin. The cells were plated and cultured at a density of $9x10^4$ cells/cm^2 for 14 DIV at 37° C in a humidified incubator with 5% CO_2 and 95% air.

4. Cell treatments

Cells were exposed to high glucose to mimic a condition of hyperglycemia. Retinal and hippocampal cells were incubated with 24.5 mM D-glucose, plus 5.5 mM or 25 mM from the culture medium, after 5 and 7 days in culture, respectively. Cells were also incubated with 24.5 mM D-mannitol, plus 5.5 mM glucose for retinal or 25 mM glucose for hippocampal cells in the culture medium, which was used as an osmotic control. Retinal cells and hippocampal neurons were exposed to high glucose for 2 and 7 days, respectively.

5. Biotinylation and purification of plasma membrane-associated proteins

Cells were washed twice with phosphate-buffered saline (PBS) supplemented with calcium and magnesium (PBS/$Ca^{2+/}Mg^{2+}$: 137 mM NaCl, 2.7 mM KCl, 1.8 mM KH_2PO_4, 10 mM Na_2HPO_4, 0.5 mM $MgCl_2$, 1 mM $CaCl_2$; pH 7.4), and then incubated with 1 mg/ml Sulfo-NHS-SS-Biotin for 30 min on ice under mild shaking in the dark.

Cells were then washed twice with PBS/Ca^{2+}/Mg^{2+} supplemented with glycine (100 mM) and incubated in the same solution for 45 min at 4ºC under mild shaking in the dark. Cells were lysed with RIPA buffer (150 mM NaCl, 50mM Tris, 5mM EGTA, 1% Triton X-100, 0.5% DOC, 0.1% SDS) supplemented with *complete-mini* protease inhibitor cocktail tablets and phosphatase inhibitors (50 mM NaF and 1 mM Na_3VO_4). Immediately, the cells were scraped, sonicated for 30s and centrifuged at 14,000 rpm for 10 min at 4°C. The supernatant was then transferred to clean tubes. The protein concentration was determined by the BCA protein assay. The concentration and the volume of the samples were equalized by adding RIPA buffer. After that, we collected a small volume of each sample (containing total protein fraction), which was transferred to clean tubes and immediately denaturated with sample buffer (100 mM Tris, 100 mM glycine, 4% SDS, 8% β-mercaptoethanol, 8 M urea and 1.5 mM sodium orthovanadate). NeutrAvidin beads were added to the remaining sample (2 µl/10 µg total protein) and incubated for 2 h at 4°C on the orbital shaker (mild shaking). After incubation with NeutrAvidin beads, samples were washed four times with RIPA buffer by centrifugation (5,400 rpm, 3 min) to remove non-

bounded proteins. After washing, beads-bound proteins were eluted with sample buffer by centrifugation (14,000 rpm, 2 min) using a collector tube with a 0.45 μm filter.

6. Western blotting

Biotinylated samples were loaded (same volume per lane) and resolved by 4-8% (v/v) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), for 60 min at 160 V in Tris-Bicine buffer (25 mM Tris, 25 mM Bicine, 1% SDS; pH 8.3). Proteins were transferred onto PVDF membranes in CAPS-metanol buffer (10 mM CAPS, pH 11, 10% methanol), for 90 min at 0.75 A (4ºC). The membranes were then blocked for 1 h at room temperature in Tris-buffered saline with Tween 20 (TBS-T) (20 mM Tris, 137 mM NaCl, 0.1% Tween 20 (v/v); pH 7.6) containing 5% (w/v) low-fat milk. After that, the membranes were incubated overnight at 4ºC with primary antibodies: anti-GluR1 (1:3,000), anti-GluR2 (1:500) and anti-GluR4 (1:250) for retinal samples and anti-GluR1 (1:6,000), anti-GluR2 (1:1,000) and anti-GluR4 (1:250) for hippocampal samples. The antibodies were diluted in TBS-T solution with 1% low-fat milk. After washing for 1 h (3 x 20 min) in TBS-T with 0.5% low-fat milk, the membranes were incubated with an alkaline phosphatase-linked secondary antibody (1:20,000) for 1 h at room temperature. The membranes were washed again with TBS-T with 0.5% low-fat milk (3 x 20 min) and the proteins were detected using the ECF system and the STORM 860 imaging system (Molecular Dynamics, Amersham Biosciences, Uppsala, Sweden). In the end, digital quantification of the densitometry of the bands was performed using Image Quant 5.0 software (Amersham). To check if the amount of loaded protein was similar between the different lanes, and if the biotinylated fraction did not comprise cytoplasmic proteins, the membranes were re-incubated with primary antibodies anti-transferrin (1:1,000) and anti-actin (1:20,000).

7. Immunostaining

Rat retinal neural cells were washed twice with PBS (137 mM NaCl, 27 mM KCl, 18 mM KH_2PO_4, 100 mM Na_2HPO_4; pH 7.4), fixed in 4% paraformaldehyde with 4% sucrose for 10 min, and rinsed with PBS* (PBS supplemented with 0.02% (w/v) sodium

azide and 0.03% (w/v) bovine serum albumin (BSA)). After that, the cells were permeabilized with 1% Triton X-100 in PBS* for 10 min. Non-specific binding of the antibodies was prevented by incubation with 10% goat serum in PBS* for 20 min. Cells were incubated with primary antibodies anti-MAP2 (1:1,000) and anti-GluR1/GluR2/GluR4 (1:300/1:250/1:200), respectively. After 2 h incubation, cells were washed with PBS* and incubated with the secondary antibodies, Alexa 488 goat anti-rabbit IgG (1:250) and Alexa 568 goat anti-mouse IgG (1:250), and with DAPI to stain cell nuclei for 1 h in the dark. Cells were rinsed and the coverslips were mounted on glass slides. All steps of this technique were performed at room temperature. The preparations were visualized with a LSM 510 confocal scanning laser microscope (Zeiss, Gottingen, Germany).

8. Statistical analysis

The results are presented as mean ± standard error of the mean (SEM). The data were analyzed using one-way analysis of variance (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 4.0 Software (GraphPad Software).

IV – Results

1. High glucose increases the surface levels of AMPA receptor subunits in rat retinal cells

In this work, we investigated the effect of high glucose on total and membrane levels of AMPA receptor subunits in retinal neural cells. Primary rat retinal neural cell cultures were exposed to 30 mM glucose or 24.5 mM mannitol (plus 5.5 mM glucose in the culture medium), which was used as an osmotic control, for 2 days. To analyse the content of AMPA receptor subunits at the plasma membrane, a biotinylation assay, followed by western blotting, were performed.

High glucose significantly increased total and membrane protein levels of GluR1 (175.7 ± 26.2% and 154.6 ± 11.5% of the control, respectively) (Figs. 1A and B) and GluR2 (138.8 ± 12.4% and 133 ± 10% of the control, respectively) (Figs. 1C and D) subunits. Regarding GluR4 subunit, the total protein content was not affected, whereas the surface levels were significantly greater (145.1 ± 15.1% of the control) than those found in control cells (Figs. 1E and F). Finally, the exposure to mannitol did not change the total and plasma membrane content of AMPA receptor subunits.

The total content of AMPA receptor subunits (GluR1, GluR2 and GluR4) was also evaluated by immunocytochemistry. In agreement with the results obtained by immunoblotting, we could observe an increase in the immunostaining for GluR1 and GluR2 (Figs. 2 and 3), but not for GluR4 subunit (Fig. 4), in the majority of neurons stained for GluR1, GluR2 and GluR4 subunits, in high glucose-treated cells. The images also show that the staining for all AMPAR subunits is more pronounced in the cell bodies than in the processes. The cells were also labelled for MAP2, which was used to identify neurons, and we found no differences in cell morphology among the different conditions.

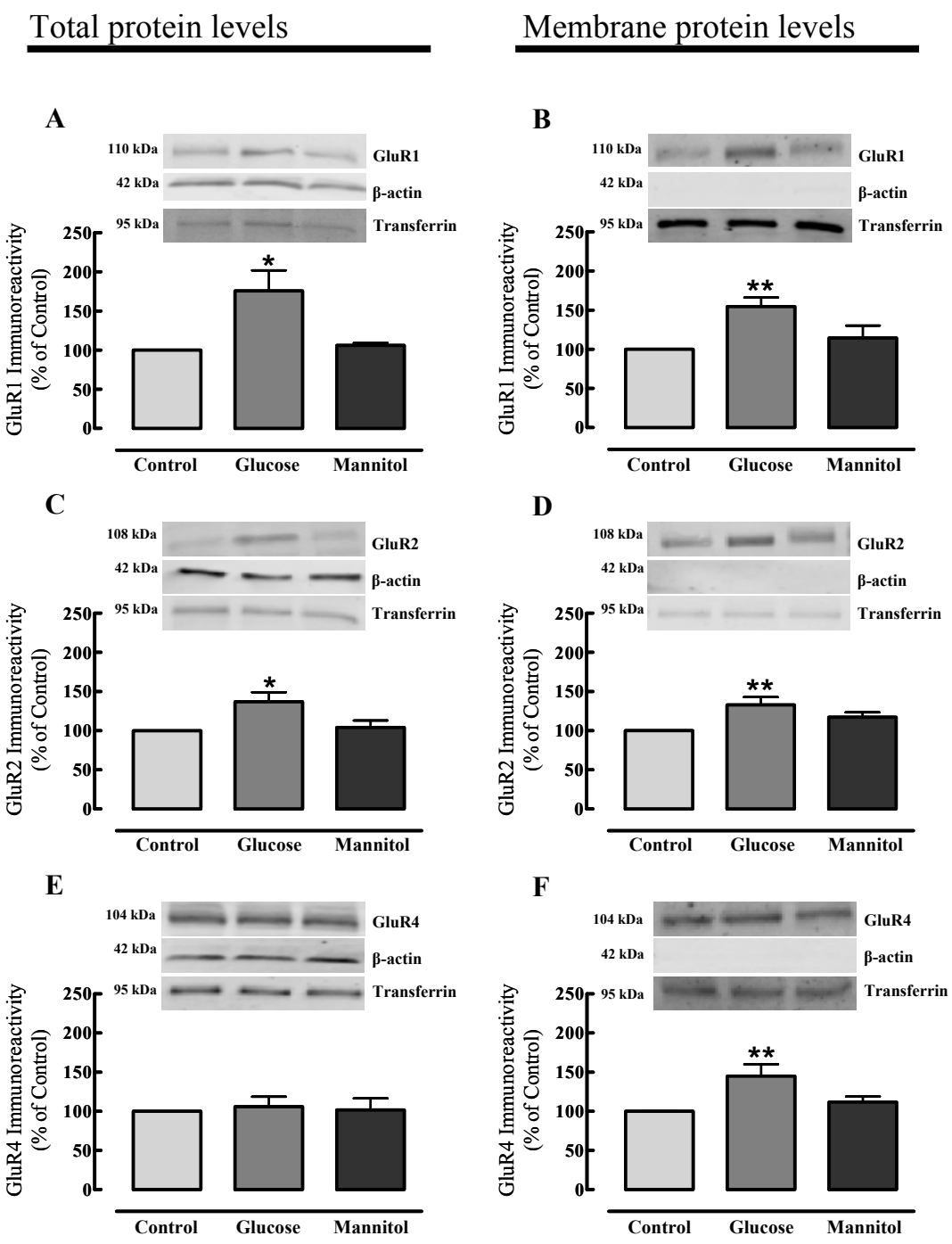

Figure 1 - High glucose increases the content of GluR1, GluR2 and GluR4 subunits at the membrane in rat retinal neural cells. The cells were exposed to 30 mM glucose and 24.5 mM mannitol (plus 5.5 mM glucose) for 2 days. The results represent the mean ± SEM of at least three independent experiments, and are presented as percentage of control. Statistical significance was determined by one-way ANOVA, followed by Dunnet's post-hoc test; *$p<0.05$, **$p<0.01$ – significantly different from control.

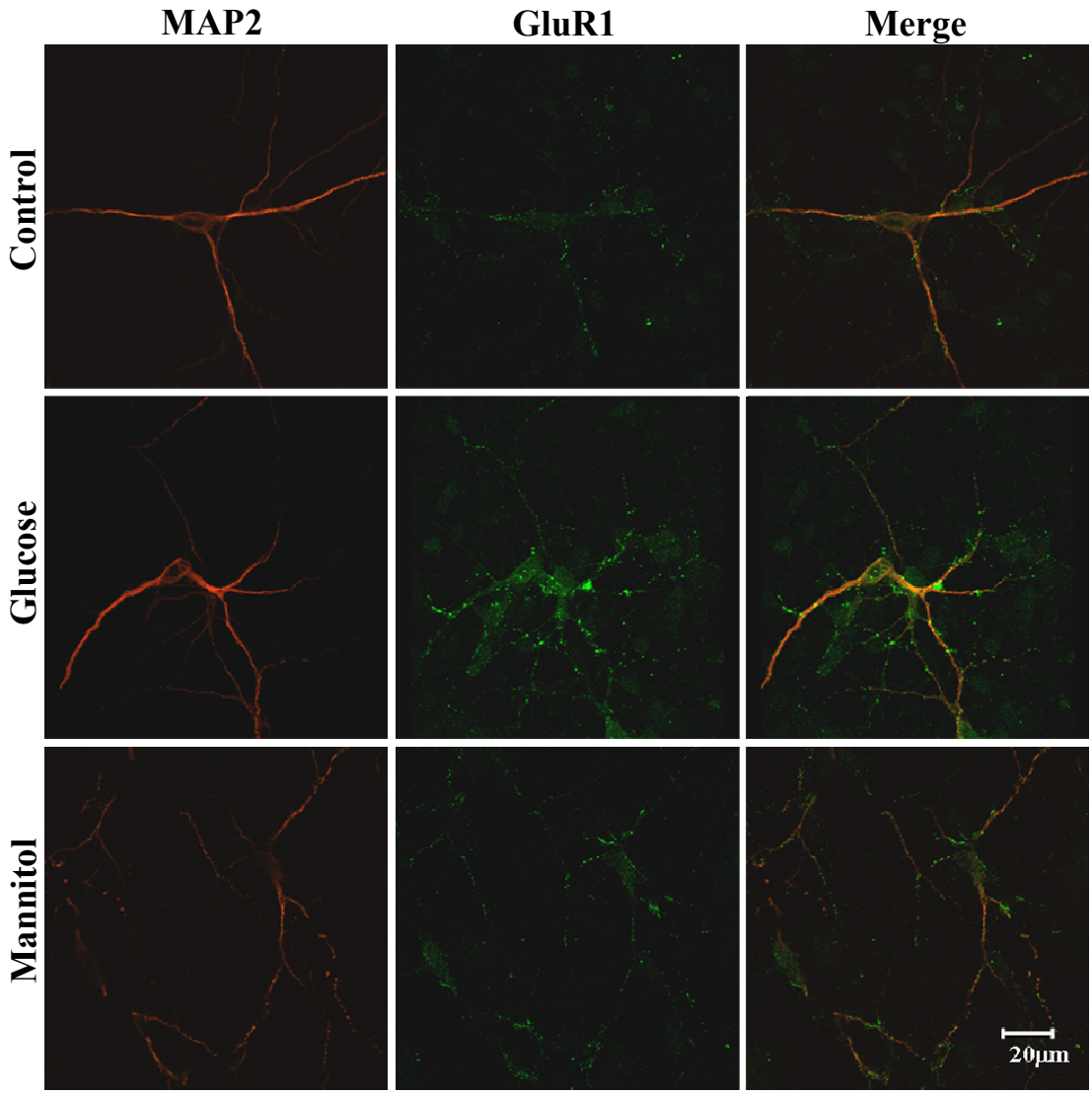

Figure 2 - High glucose increases GluR1 immunoreactivity in retinal neurons. Primary rat retinal neural cell cultures were exposed to 30 mM glucose or 24.5 mM mannitol (plus 5.5 mM glucose) for 2 days, and then cells were immunolabeled for GluR1 (630x magnification).

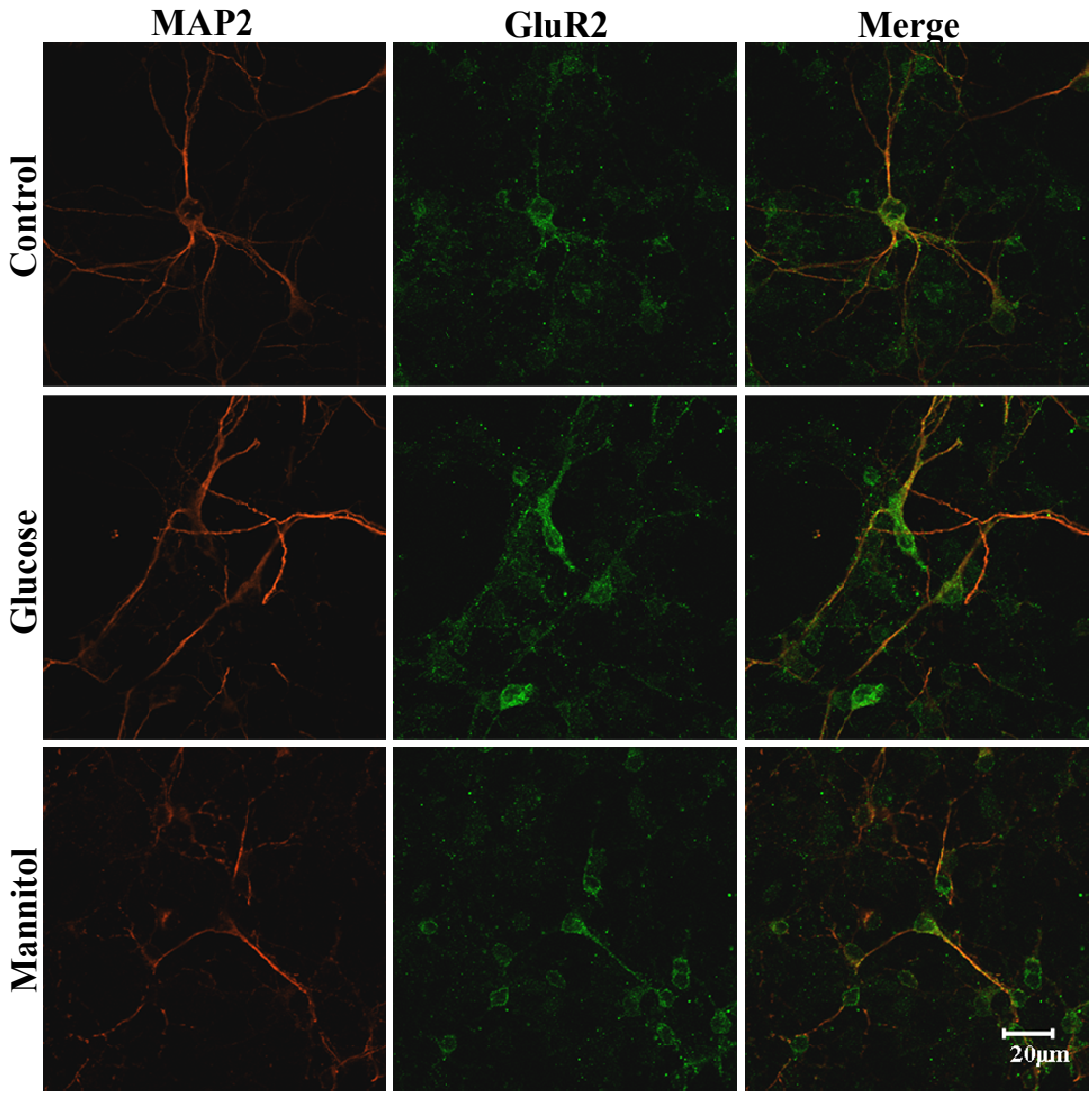

Figure 3 - High glucose increases GluR2 immunoreactivity in retinal neurons. Primary rat retinal neural cell cultures were exposed to 30 mM glucose or 24.5 mM mannitol (plus 5.5 mM glucose) for 2 days, and then cells were immunolabeled for GluR2 (630 x magnification).

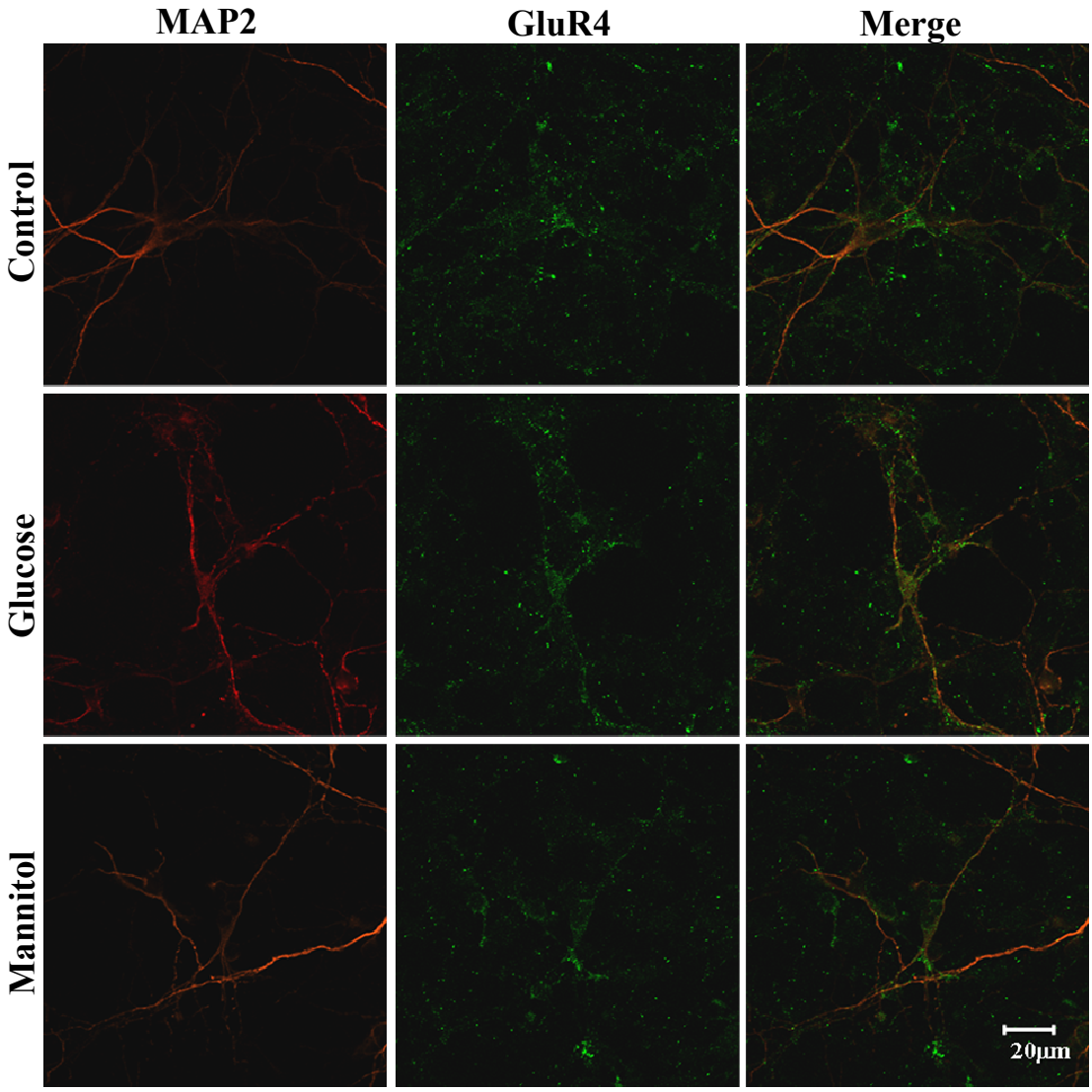

Figure 4 - High glucose does not change GluR4 immunoreactivity in retinal neurons. Primary rat retinal neural cell cultures were exposed to 30 mM glucose or 24.5 mM mannitol (plus 5.5 mM glucose) for 2 days, and then cells were immunolabeled for GluR4 (630 x magnification).

2. High glucose does not alter the total and surface levels of AMPA receptor subunits in rat hippocampal neurons

Since we found that high glucose could affect the levels of AMPA receptor subunits in retinal cells, we also evaluated the effect of high glucose on the total and surface expression of AMPA receptor subunits in rat hippocampal cell cultures by using a biotynilation assay and immunoblotting. It has been documented that diabetes impairs memory and cognitive processes, and it is well established that hippocampus and ionotropic glutamate receptors are involved in these processes (Biessels et al., 1996; Chabot et al., 1997).

Primary cultures of hippocampal neurons were exposed to 50 mM glucose or 24.5 mM mannitol (plus 25 mM glucose) for 7 days. Contrarily to what was observed in retinal cell cultures, in hippocampal cultures high glucose exposure did not change significantly the total and surface levels of GluR1, GluR2 and GluR4 AMPAR subunits. Likewise, the exposure to mannitol did not affect the content of these proteins (Fig. 5).

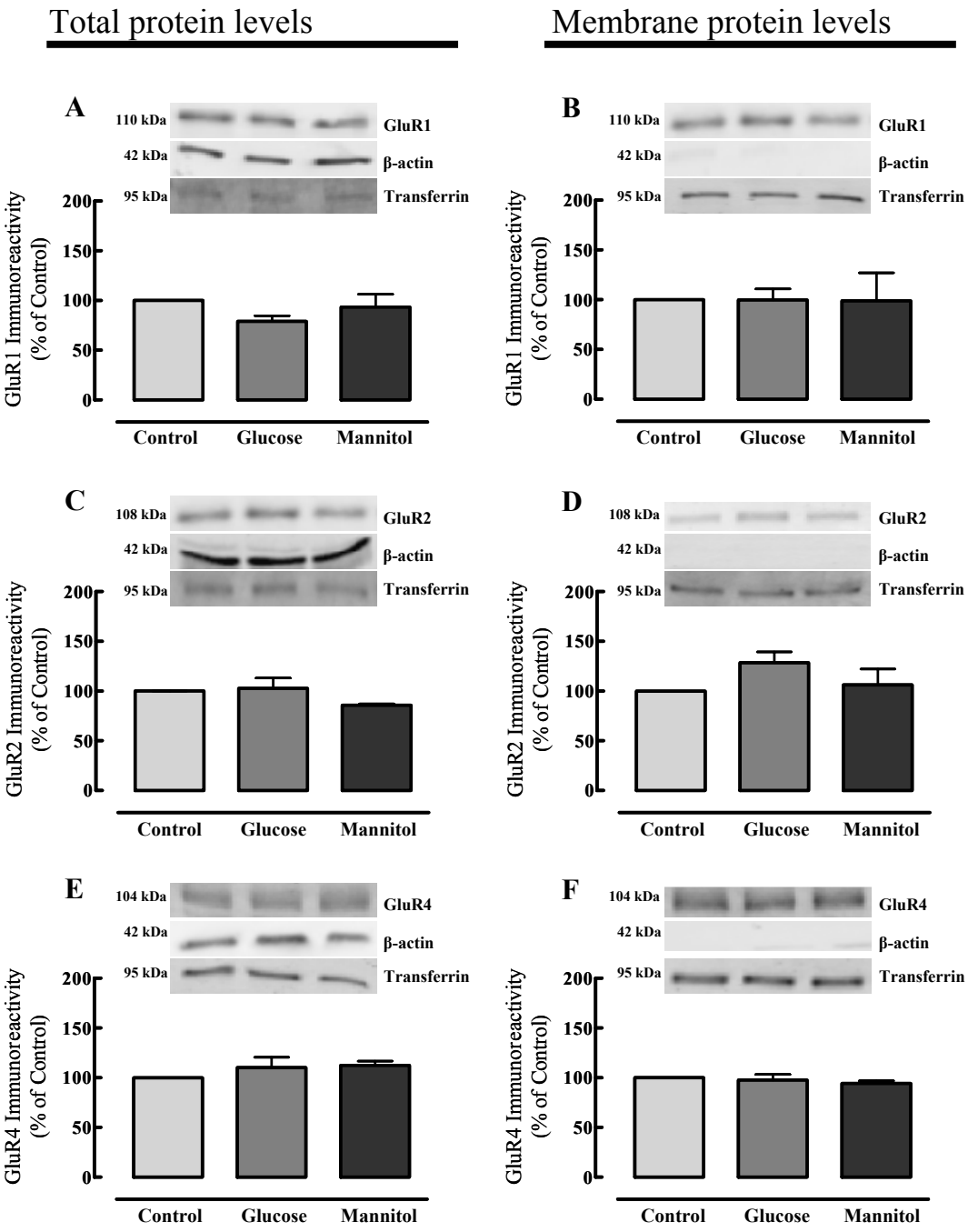

Figure 5 - Elevated glucose does not change total and membrane content of AMPAR subunits in rat hippocampal neurons. The cells were exposed to 50 mM glucose and 24.5 mM mannitol (plus 25 mM glucose) for 7 days. The results represent the mean ± SEM of at least three independent experiments, and are presented as percentage of control. Statistical significance was determined by one-way ANOVA, followed by Dunnet's post-hoc test.

V – Discussion

Diabetic retinopathy is a frequent complication of diabetes *mellitus* and a leading cause of vision loss and blindness in working-age adults, particularly in western countries (Kempen et al., 2004). Microvascular dysfunction is still considered the hallmark of diabetic retinopathy. However, increasing evidence has demonstrated the existence of neuronal dysfunction and degeneration and impaired glutamate metabolism in the retina in the early stages of diabetes (Barber, 2003; Fletcher et al., 2005). Diabetes has also been associated with a moderate cognitive impairment, characterized by a lowered cognitive performance and defects in hippocampal-mediated memory processes (Biessels et al., 1996; Gispen and Biessels, 2000; Biessels and Gispen, 2005).

AMPAR have a predominant role in glutamatergic synaptic transmission in the retina and hippocampus. In the retina, AMPAR are mainly located in the plexiform layers (Brandstatter and Hack, 2001). In the hippocampus, they are abundantly expressed in the pyramidal cell layer and *dentate gyrus* (Ozawa et al., 1998). Recent evidences demonstrated that high glucose and diabetes can affect the content of ionotropic glutamate receptors in the retina (Ng et al., 2004; Santiago et al., 2006b; Santiago et al., 2008) and hippocampus (Gardoni et al., 2002), which might have consequences on glutamatergic transmission and therefore underlie retinal and cognitive dysfunction in diabetes. In fact, recent studies also suggest an abnormal expression of multiple glutamate receptors in different tissues of diabetic rats. The mRNAs coding for several AMPA (GluR1, GluR2 and GluR3) and NMDA receptor subunits (NR2A and NR2B), and for mGluRs (mGluR1 and mGluR5) are upregulated in the dorsal horn of the lumbar spinal cord of streptozotocin-induced diabetic rats (Tomiyama et al., 2005). Likewise, it was reported an increase in the gene expression of NR2B and mGluR5 glutamate receptor in the cerebral cortex of diabetic rats (Joseph et al., 2008). These findings further indicate that hyperglycemia alters the expression of glutamate receptors, which might have consequences in glutamatergic neurotransmission.

Based on these evidences, mainly on those obtained in retinal cell cultures in our laboratory, and since hyperglycemia is considered the primary pathogenic factor for the development of diabetic retinopathy and memory and cognitive deficits in diabetes (DCCTResearchGroup, 1993; Trudeau et al., 2004), we further investigated the effect of

elevated glucose (to mimic hyperglycemic conditions) on the content of AMPAR subunits in retinal cells. Also, we hypothesized that high glucose could affect the content of AMPAR subunits at the plasma membrane. Therefore, we analysed the effect of 2 days exposure to high glucose (in previous studies performed in our laboratory, retinal cells were exposed to high glucose for 7 days), which can be also considered a long-term incubation, on the total and plasma membrane levels of GluR1, GluR2 and GluR4 (previous studies in retinal cell cultures were focused on GluR2 subunit). In addition, since the hippocampus can be also affected by hyperglycemia, we performed similar studies in hippocampal cell cultures.

In this study, we showed that high glucose differentially affects AMPAR subunits protein expression in retinal and hippocampal cell cultures, suggesting that retinal neurons are more susceptible to hyperglycemia than hippocampal neurons. The effect of high glucose on AMPAR subunits content and localization in retinal cells was detected after two days exposure, whereas in hippocampal cultures the exposure to high glucose for seven days did not affect AMPAR content and localization. Previous studies (Santiago et al., 2006b) demonstrated that the content of ionotropic glutamate receptor subunits, including AMPAR, is altered when retinal cell cultures are exposed to high glucose for seven days. Actually, we cannot exclude the possibility that longer periods of exposure to high glucose could induce changes in AMPAR subunits content and distribution in hippocampal cell cultures. Also, it is important to emphasize that diabetic patients undergo retinal changes much earlier than cognitive and memory deficits, which may somehow indicate that brain, and particularly in hippocampus, are more resistant to hyperglycemia.

Our findings also demonstrate that total and surface contents of AMPAR subunits are changed in retinal cells exposed to high glucose for two days. These changes were not due to the increase in osmolarity, since mannitol, the osmotic control, had no significant effects. Similarly, previous findings in our laboratory showed that exposure of retinal cell cultures to high glucose, for seven days, decreases GluR1 and increases GluR2 subunits levels (Santiago et al., 2006b). The present results indicate that shorter exposures are also able to induce changes in AMPAR subunits in retinal cells. However, Santiago and colleagues reported a decrease in the total levels of GluR1 and we report an increase, which suggests that the content and localization of AMPAR subunits depend on exposure time to hyperglycemic conditions.

Here, we evaluated, for the first time, the effect of high glucose on membrane expression of AMPAR subunits. In retinal cells, the increase in the total content of GluR1 and GluR2 was accompanied by an increase in the levels of these subunits at the membrane. Possibly, the increase in the total content could consequently determine the increase at the level of plasma membrane. However, the trafficking of AMPAR to the membrane that is regulated by numerous proteins and phosphorylation events may also be altered by elevated glucose, and therefore must be investigated. It is important to mention that changes in GluR2 subunit distribution may be particularly relevant, because this is the most expressed subunit in adult rat retinas and in their presence AMPAR are impermeable to Ca^{2+} (Swanson et al., 1997). Actually, previous findings in our laboratory suggested, although indirectly, that the GluR2 subunit content is increased at the plasma membrane in cultured retinal neurons (Santiago et al., 2006b). This increase may be viewed as a protective mechanism against Ca^{2+} overload and consequent cell degeneration. Furthermore, an increase in GluR2 subunit was also detected in the plexiform layers in the retinas of diabetic humans (Santiago et al., 2008). In fact, another study reported a marked increase in GluR2/3 immunoreactivity in the retinas of diabetic rats, particularly in the inner and outer plexiform layers and an increase in the immunoreactivity of calcium-binding proteins, calbindin and parvalbumin (Ng et al., 2004). The increased levels of these proteins may function as buffering reservoirs to maintain the Ca^{2+} homeostasis protecting these cells against the damaging effects of excessive calcium influx during overexcitatory activity.

We also found that high glucose did not affect the total protein levels of GluR4 in retinal cells, whereas surface levels increased. Thus, elevated glucose is probably influencing the trafficking events of this subunit. However, we do not know yet which steps of AMPAR cycling might be affected. To understand how high glucose could affect AMPAR trafficking, it will be important to study the interaction of AMPAR subunits with several proteins, namely proteins that are responsible for the insertion and stabilization of AMPAR in the synapse, such as PICK 1 and NSF, and proteins that are involved in AMPAR endocytosis, such as AP2 protein.

Since retinal cell cultures are mixed cultures constituted by neurons and glial cells, and because AMPAR are also present in both cell types (reviewed by Verkhratsky and Steinhauser, 2000), we cannot exclude the possibility that glial cells could be contributing

for the changes in AMPAR subunits content demonstrated by western blotting. Therefore, by immunocytochemistry experiments and using a MAP2 antibody as a neuronal marker, it was possible to observe different staining intensities for AMPAR subunits in retinal neurons, among the conditions studied. In particular, and accordingly to western blotting experiments, in retinal neurons we found an increase in immunostaining intensity for GluR1 and GluR2 subunits, but not for GluR4. Mannitol, as expected, did not induce significant changes. However, using this technique, it was not possible to perceive the increases of those subunits at the plasma membrane level. It is also important to mention that MAP2 is not expressed, or at least not detectable, in all neurons. In a future approach, it will be indispensable to use other antibodies, against beta III tubulin, to stain retinal neurons, and glial fibrillary acidic protein (GFAP) to distinguish neurons from glial cells.

Several studies over the past decades have shown that long-term potentiation and consequently memory impairments in diabetic animals might be due to abnormalities in postsynaptic glutamatergic receptors (Biessels and Gispen, 2005). In this *in vitro* study, in hippocampal neurons, we did not observe any significant changes in total or membrane levels of AMPAR subunits among the three conditions tested, at least for a period of seven days exposure to high glucose. However, since it was reported that NMDA receptors expression was down-regulated in postsynaptic densities from the hippocampus of streptozotocin-induced diabetic rats (Gardoni et al., 2002), it would be important to check if the expression and distribution of other ionotropic glutamate receptors subunits could be altered in high glucose-treated cells.

In this work, we mimicked hyperglycemic conditions using *in vitro* models, however, in the future, it would be important to use an animal model of diabetes, to evaluate whether the membrane and synaptic expression of AMPAR subunits is altered in the retinas and hippocampus of diabetic animals. To perform that, it will be necessary to obtain synaptoneurosome preparations, and then isolate the membrane fraction, or prepare postsynaptic density-enriched fractions (Hollingsworth et al., 1985; Williams et al., 2007).

VI – Conclusions

This work allowed us to draw the following main conclusions:

1. High glucose can increase the total and membrane protein levels of AMPAR subunits in retinal neural cells, and this effect is not due to an increase in osmolarity. This result suggests that glutamatergic neurotransmission might be impaired in the retina of diabetic patients or animals.

2. In contrast, in hippocampal neurons, the total and membrane protein levels of AMPAR subunits appear not to be significantly affected by elevated glucose, suggesting that hippocampal neurons are more resistant to hyperglycemia-associated stress than retinal neurons, which may somehow explain why memory and cognitive deficits occur later than retinal dysfunction in diabetic patients.

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